Practical aspects of acquiring high-quality protein SSNMR spectra

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Winter School on Biomolecular Solid State NMR

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Disclaimer

Top Ten Tips for Producing ^{13}C ^{15}N Protein in Abundance

Deborah A. Berthold, Victoria J. Jeisy, Terry L. Sasser, John J. Shea, Heather L. Frericks, Gautam Shah, and Chad M. Rienstra
Departments of Chemistry and Biochemistry
University of Illinois at Urbana-Champaign

What could be easier than overexpressing an E.coli protein in E.coli? You don't have to be an old hand at protein expression to know that this can often be more difficult than it sounds. We tested our skills recently with DsbA, a 20 kDa protein that catalyzes disulfide bond formation in the E.coli periplasm. The wildtype DsbA expressed well in LB medium, and also in a Bio-Express-supplemented ^{13}C ^{15}N labeling medium. Likewise, the DsbA C33S mutant expressed well in LB. But when we first tried to label C33S, our luck ran out-- we saw no expression at all. Today we are producing ^{13}C ^{15}N DsbA C33S at a yield

- **If in doubt, trust the application note.**
- **Deb wrote it.**

Outline

- General Considerations for Efficient Production of Labeled Proteins: DsbA
- Expression of Membrane Proteins: DsbB
- Dilution of the ^{13}C Reservoir: GB1
- Dilution of the ^{1}H Reservoir: GB1

Expressing Proteins in *E. coli*: Growth Medium

Our medium for ^{13}C ^{15}N uniform labeling:

- **Phosphate-buffered, with 2 mM $MgSO_4$**

  Studier (2005) Prot. Exp. Purif. 41, 207

  → 2 mM vs 1 mM Mg^{2+} can sometimes double final cell density !

- **Uses ^{13}C ^{15}N BioExpress, at 10 ml/L (CIL)**

  10% of the full dose, as supplement to medium
  Holdeman & Gardner, *J. Biomol. NMR* 21: 383 (2001)

- **Includes trace metals**

  → Improves protein yield even in presence of BioExpress !

- **Contains U-^{13}C-glucose and ^{15}N-NH_4Cl (CIL)**

Synuclein Expression

Preparation of α-synuclein fibrils for solid-state NMR: Expression, purification, and incubation of wild-type and mutant forms

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Received 30 November 2005, and in revised form 12 February 2006

Expression and purification of U-^{13}C,^{15}N AS

To preclude any potential interaction of 6-His or other purification tags with the AS protein, a tag-free expression and purification system was developed. AS was expressed from pET28a-AS in *E. coli* BL21(DE3). Optimization of induction conditions (0.5 mM IPTG, 3 h induction) resulted in an increase in yield from ~2 to 5 mg/L of culture. Use of M9 minimal medium allowed complete incorporation of the stable isotope labels ^{13}C and ^{15}N from [^{13}C]glucose and [^{15}N]ammonium chloride. Supplementation of the medium with 10 ml of ^{13}C,^{15}N-Bioexpress resulted in an increase in yield of AS from ~5 to ~30 mg/L. This corresponds to 10% of the recommended concentration of Bioexpress, as reported previously to enhance yield of isotopically labeled proteins for NMR studies [19].

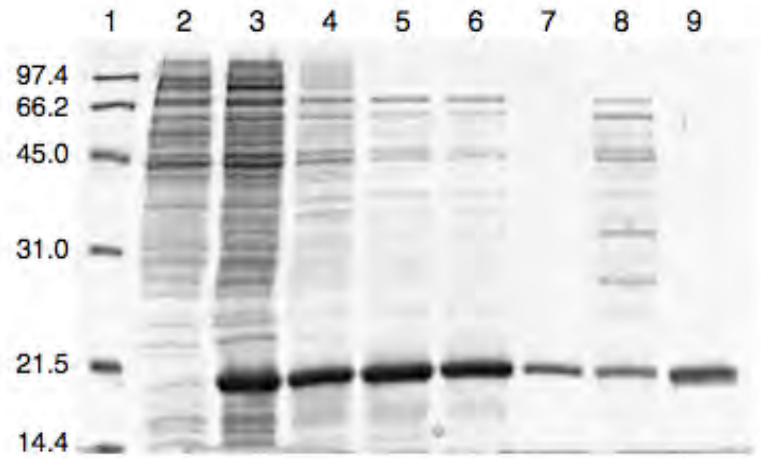
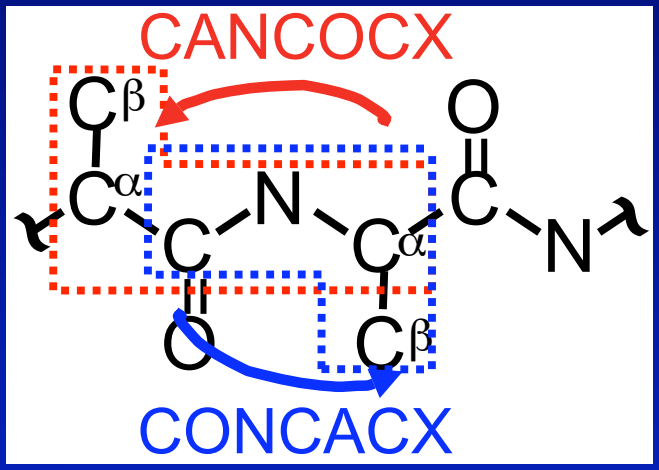


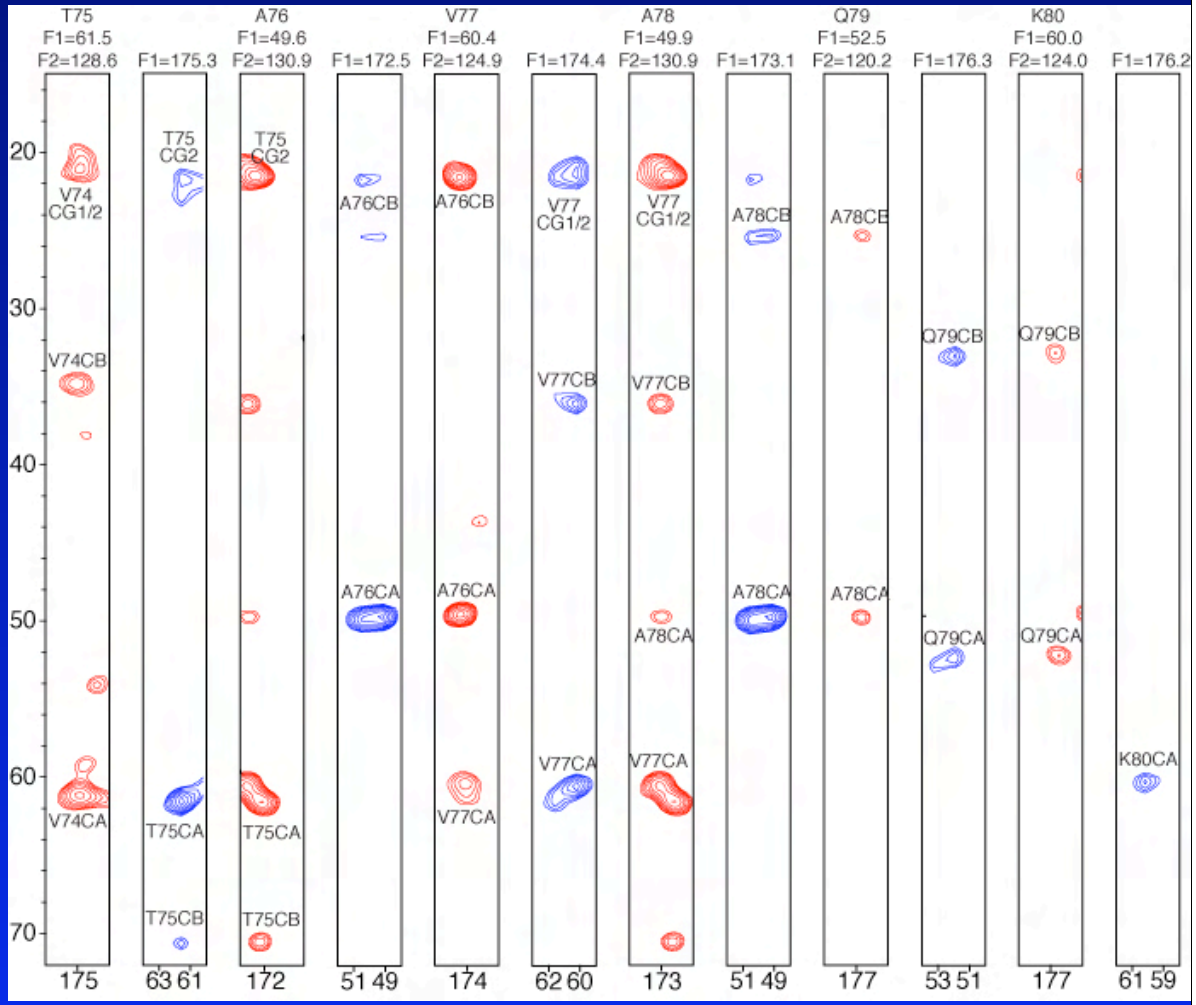
Fig. 1. SDS–PAGE analysis of a representative purification of AS. Data shown is from A30P AS. Lane 1, protein molecular weight markers, labeled in kDa; lane 2, uninduced total protein; lane 3, 3 h post-induction with IPTG; lane 4, supernatant remaining after cell lysis; lane 5, supernatant after heat purification; lane 6, ammonium sulfate precipitated protein; lane 7, primary AS-containing fractions from hydrophobic interaction chromatography; lane 8, secondary peak from hydrophobic interaction chromatography; lane 9, AS after size exclusion chromatography.

- **6X improvement with BioExpress at the supplementary level (10% of a full dose)**
- ***Protein Expr. Purif.* 2006**, *48*, 112-117.

4D Data: AS fibrils



Kathryn Kloepper



- 500 MHz, 1 umol, 42 h data acquisition
- Enables more complete and reliable assignments

Expressing Proteins in *E.coli:* Strains, Plasmids and Promoters

- Select your expression system using these two considerations --
  - Minimal leakiness of promoter
  - Good coupling of rates of transcription, translation, and post-translational events

Expressing Proteins in *E. coli*: Timing of Induction and Harvest

- **Induction of expression with IPTG:**
  - **Typically, protocols call for induction at a cell density of A600 = 0.8**
  - **We find at induction at 80% the maximal cell density for a given medium (A600 = 1.6, for instance) can often give a greater yield**
- **Harvest**
  - **A lower-temperature induction (25°C) may require a longer induction period**
  - **Yield can sometimes be improved by harvesting at 12-20 hours post-induction**

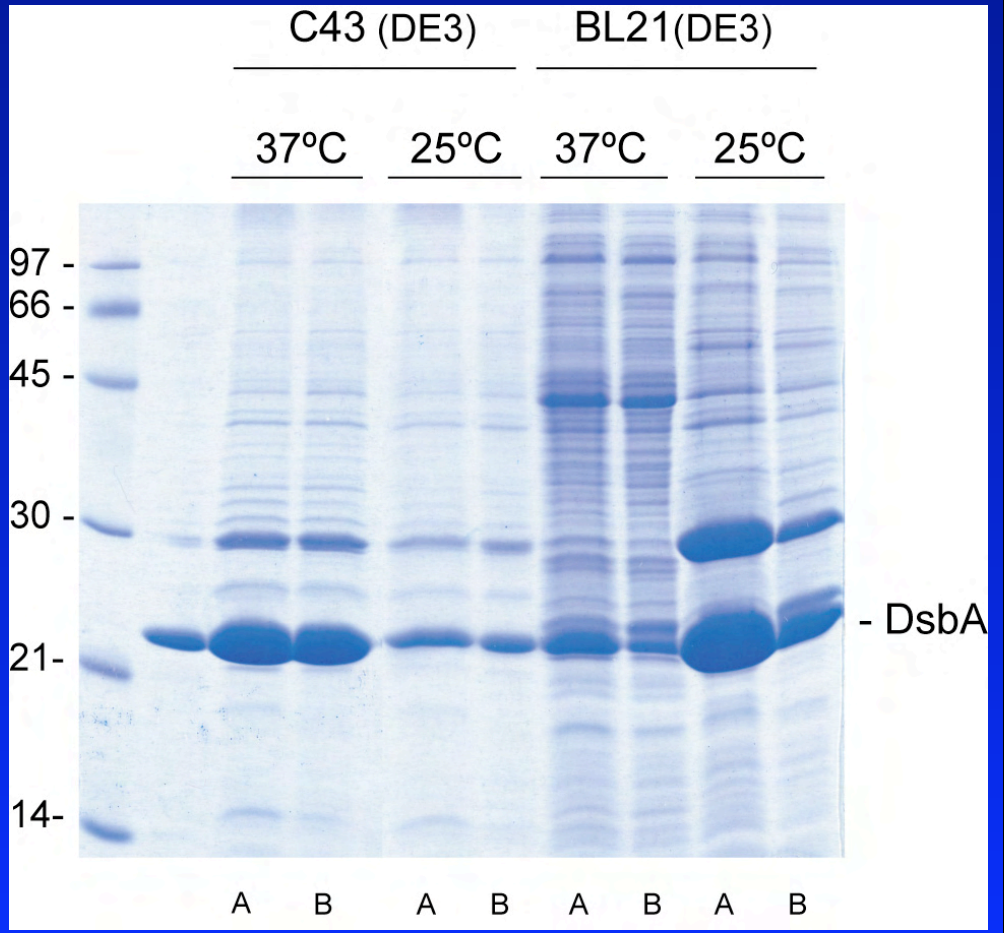
Expressing Proteins in *E. coli*: Growth Conditions

- Aeration: 250 ml in a 2 L baffled flask
- Induction Temperature

DsbA expression was poor using the standard method: BL21 (DE3) induced at 37°C.

By either lowering to 25°C or changing to C43 (DE3) we saw dramatic improvement.

This is probably due to a better coupling of transcription, translation, and post-translational events.



Disulfide Bond Formation in *E. coli*

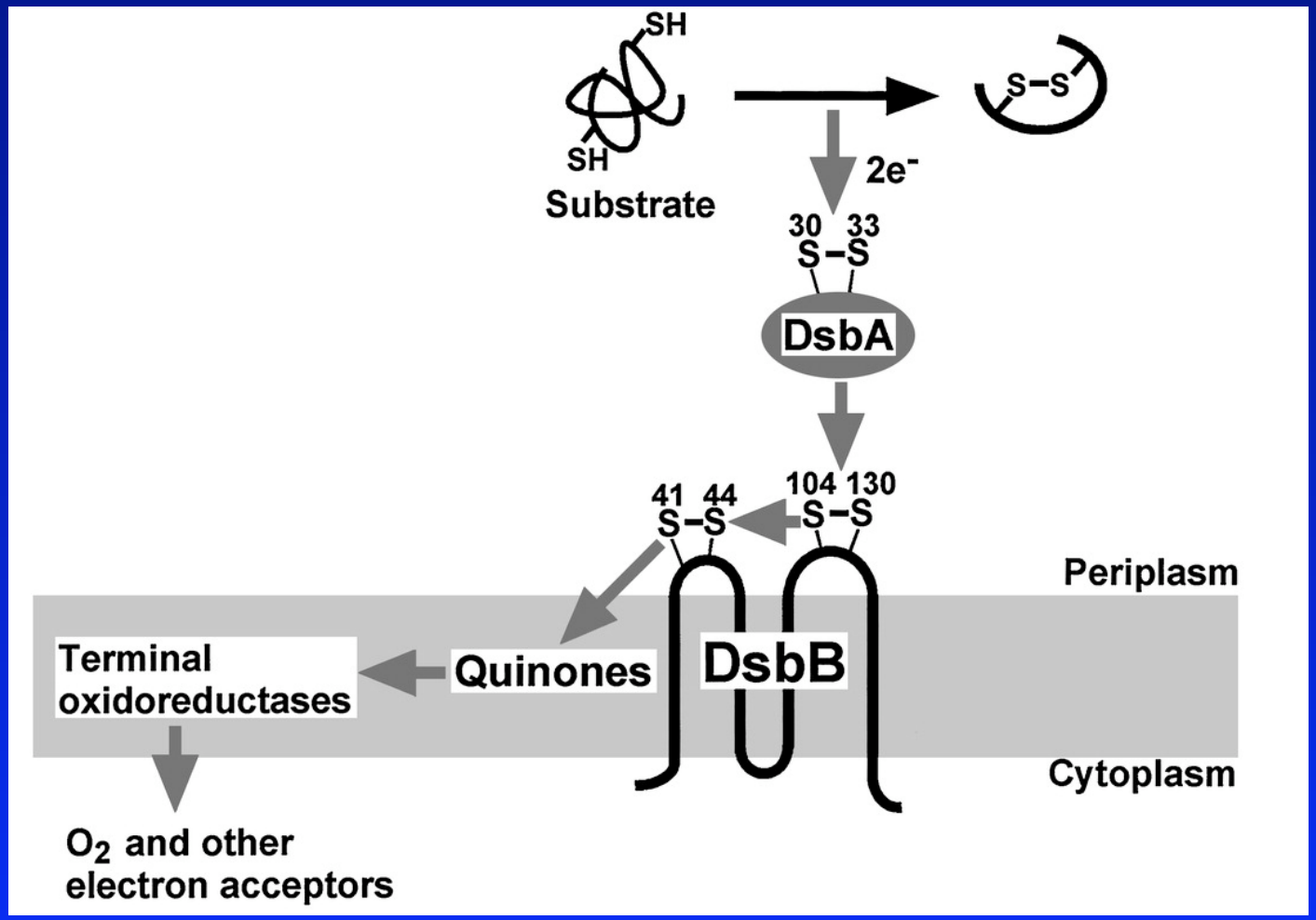
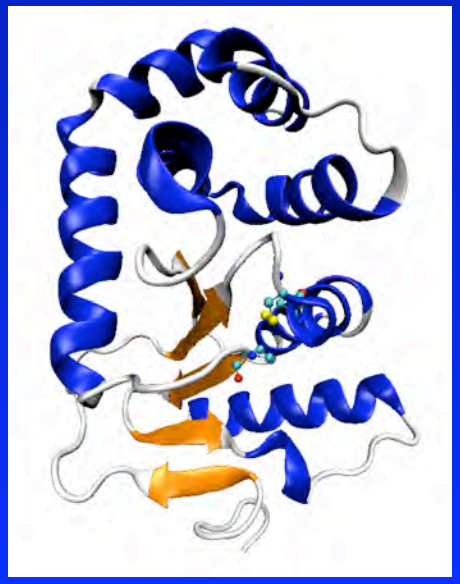


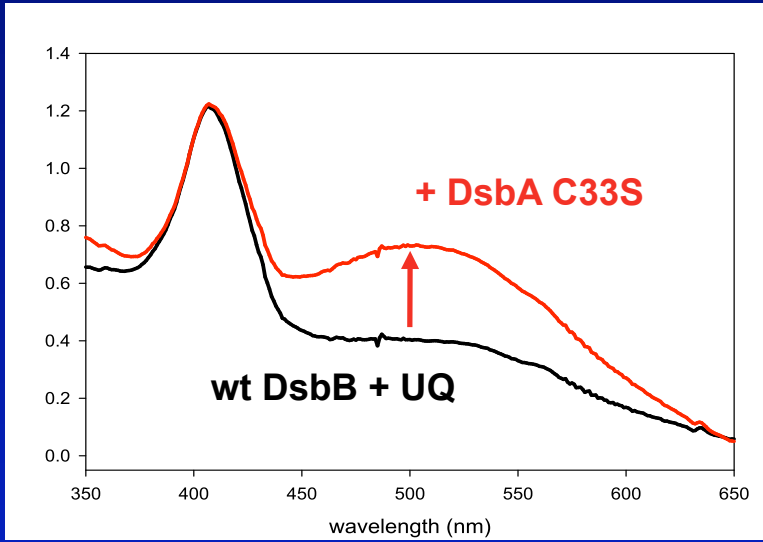
Figure 2, *Annu. Rev. Biochem.* 2003, 27: 111-135

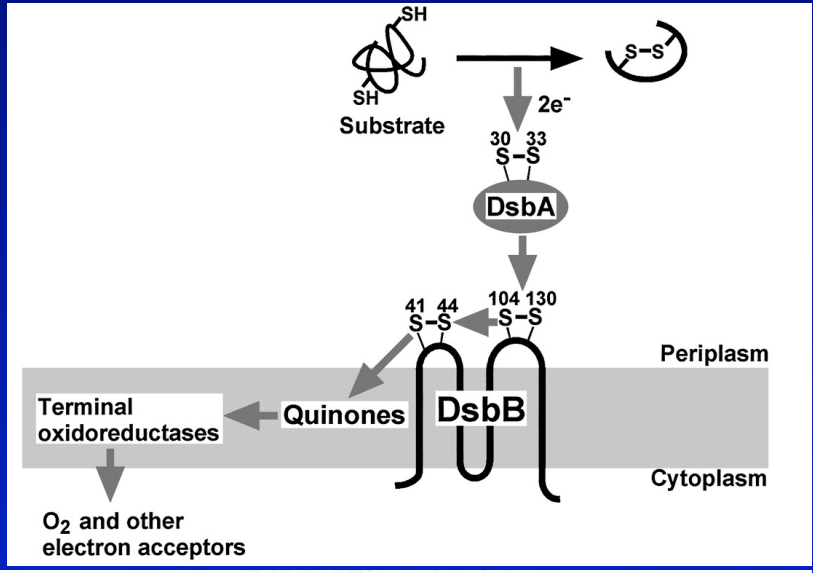
DsbA Nanocrystallization

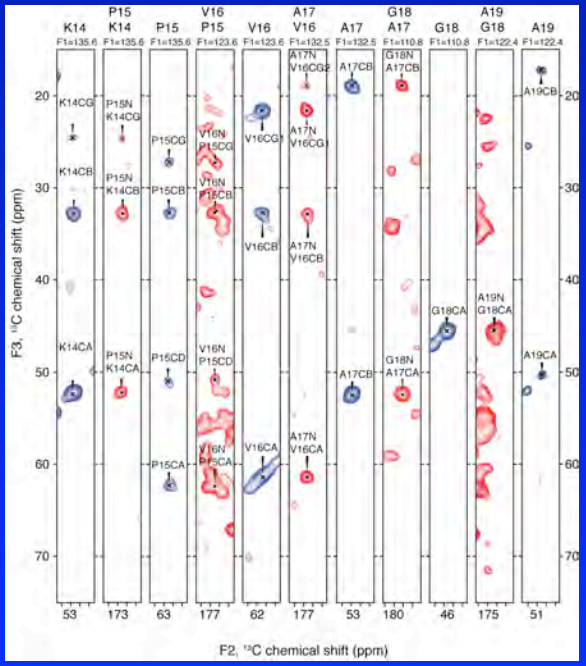
- Start with concentrated protein solution
  - ~2 mM DsbA (45 mg/mL)
  - 10 mM MOPS, pH 7
- Add 1 vol precipitant
  30% PEG 8000,
  0.1 M cacodylate,
  1.5% MPD; pH 6.5
- Dialyze against same for overnight growth of "Nanocrystals"
- Pellet into NMR rotor

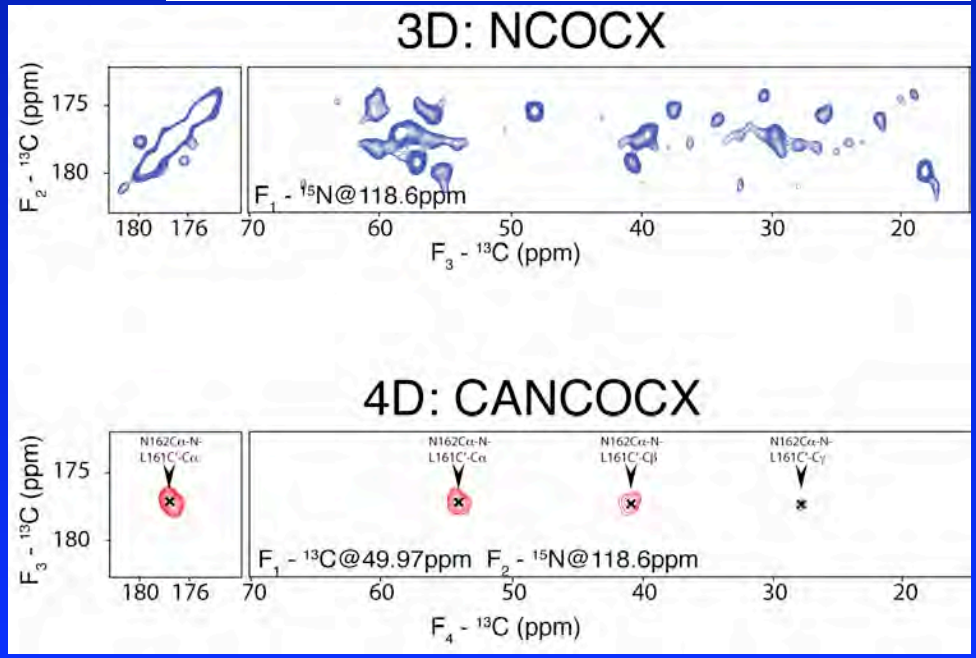


DsbA-DsbB Complex





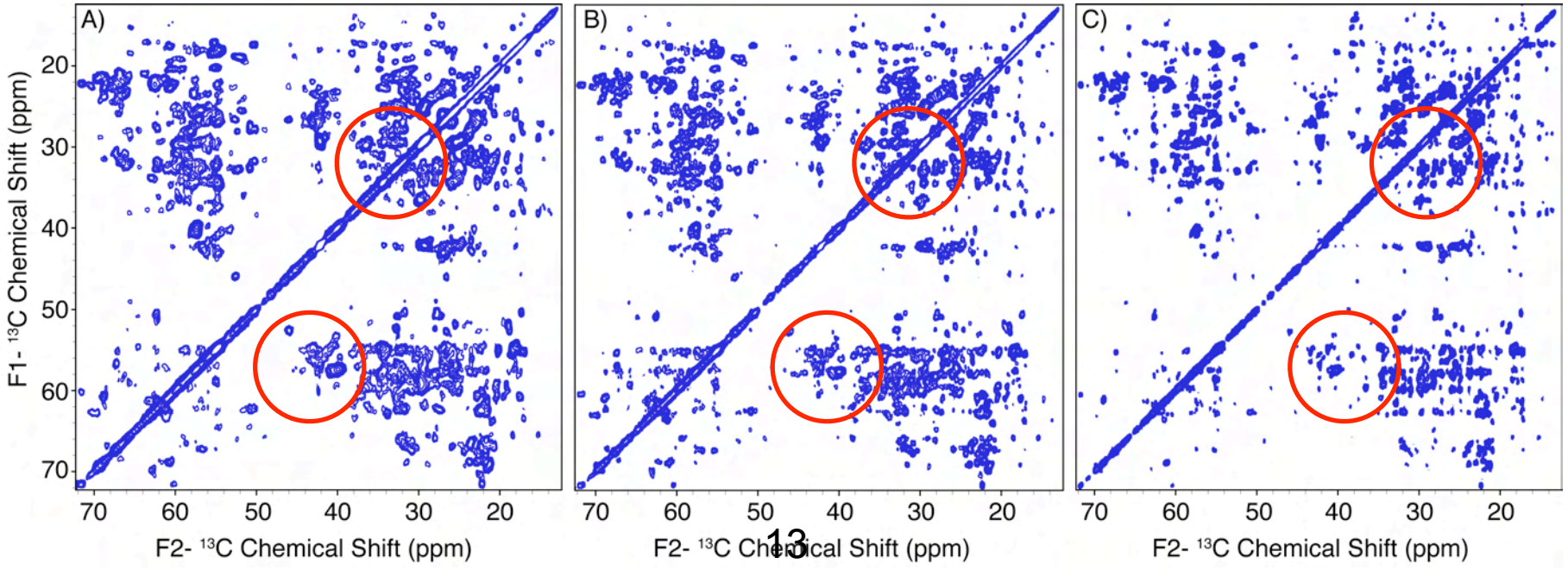




DsbA, 21 kDa Microcrystals

Microcrystalline
Linewidths ~ $1/B_0$
Quadratic benefit in 2D ^{13}C-^{13}C spectra

F(^{1}H):	500 MHz	750 MHz	900 MHz
DARR:	50 ms	100 ms	200 ms

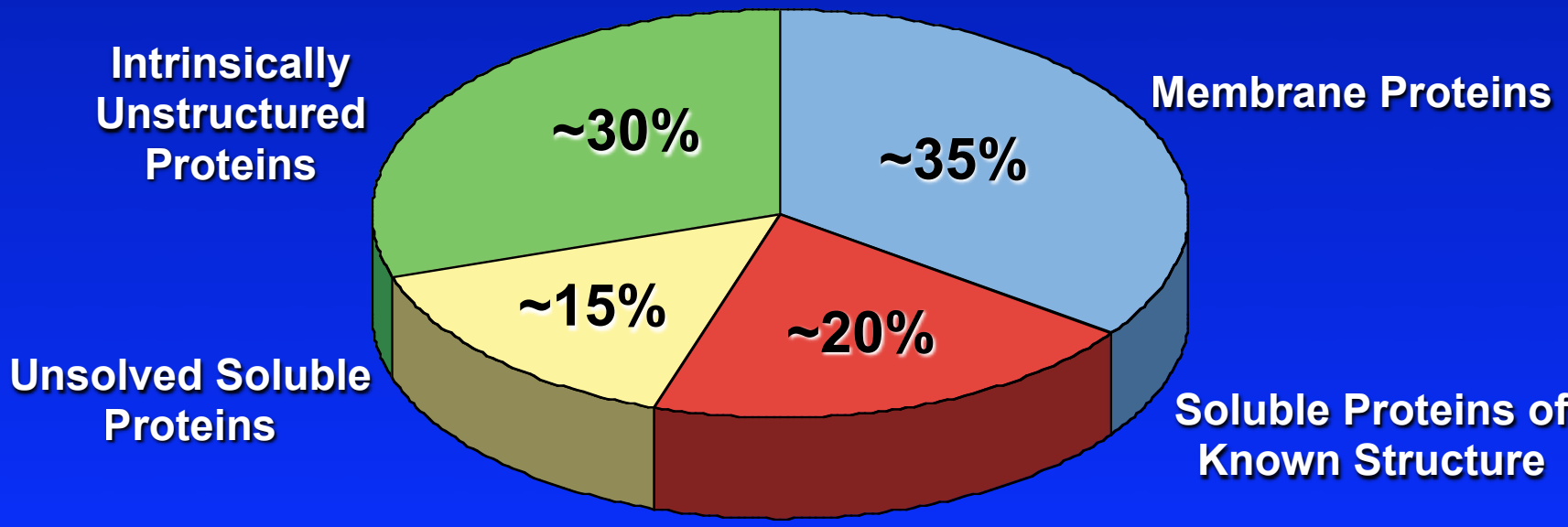


Outline

- **General Considerations for Efficient Production of Labeled Proteins: DsbA**
- **Expression of Membrane Proteins: DsbB**
- **Dilution of the ^{13}C Reservoir: GB1**
- **Dilution of the ^{1}H Reservoir: GB1**

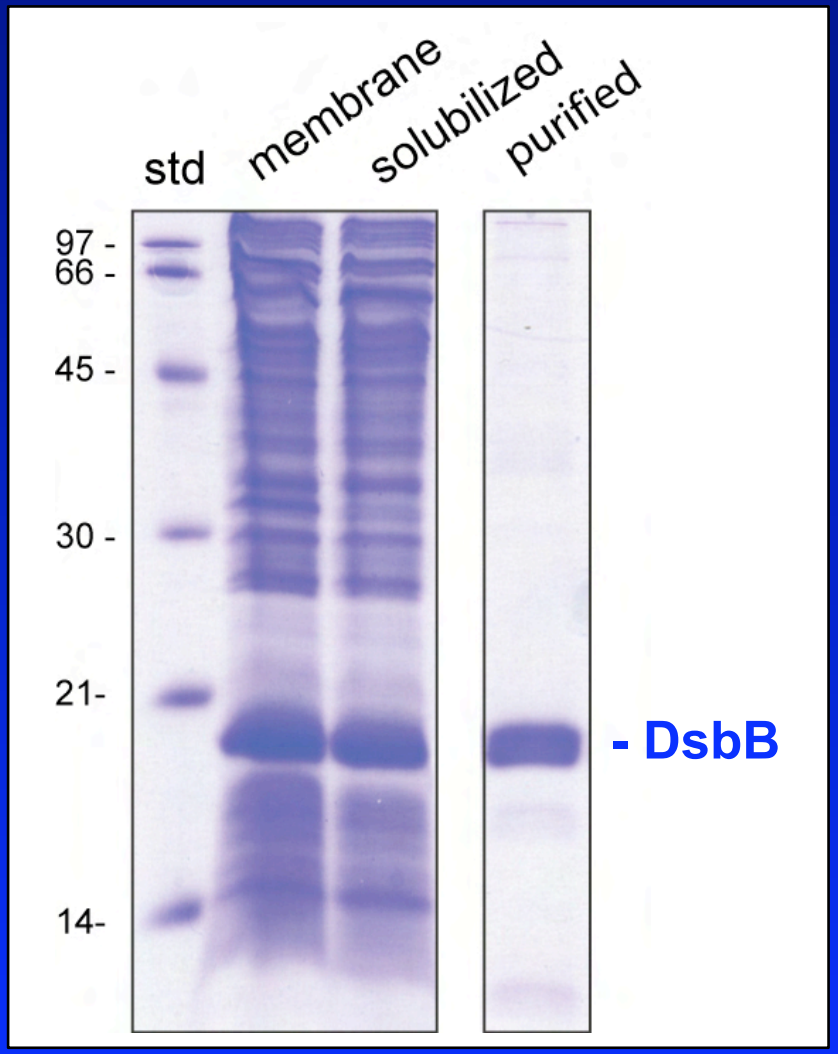
Underrepresented Protein Structures

- **Protein Data Bank: ~40K structures**
- **Not representative of sequenced genomes**
  - **Membrane proteins are ~35% of ORFs**
  - **Only 100 unique MP structures (~10 mammalian)**
  - **Only one GPCR (rhodopsin)**



DsbB Expression

- Original published expression method used sub-saturating concentrations of IPTG to try to "slow" transcription
  - But IPTG acts as on/off switch, so transcription level cannot easily be titrated
  - Difficult to reproduce
- Instead, we:
  - Moved the plasmid to *E.coli* strain C43 (DE3)
  - Dropped the induction temperature from 37°C to 25°C
  - Induced with saturating level (0.2 mM) of IPTG
  - Increased the harvest time from 4 hrs to 20 hrs post-induction



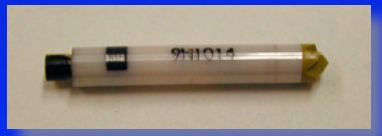
Preparation of DsbB Sample

- Uniformly label DsbB with ^{13}C and ^{15}N by expressing DsbB in isotopically enriched minimal medium
  - Yield is ~10 mg/L after optimization
- Solid sample preparation as follows:

Remove detergent by dialysis

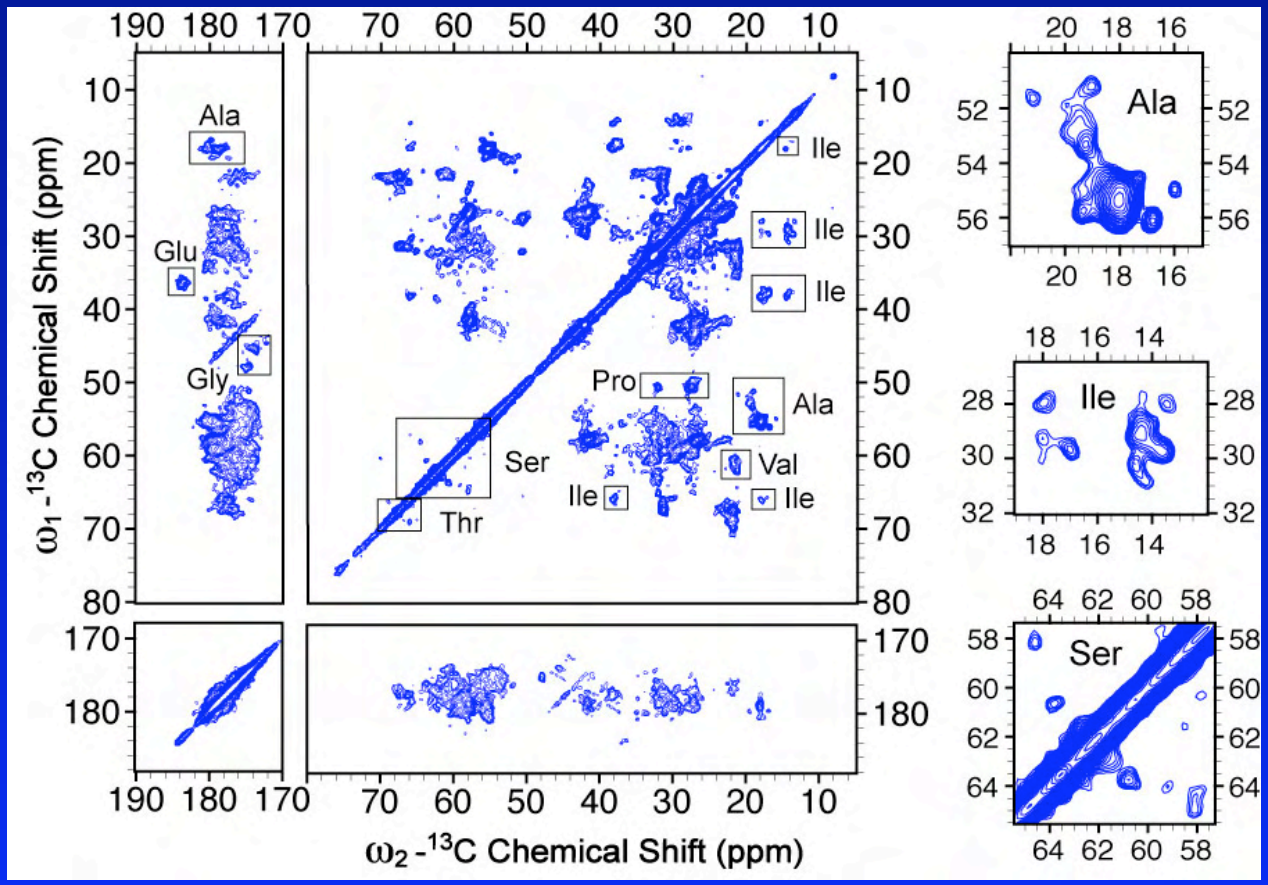
Collect protein by ultracentrifugation

Pack into 3.2 mm SSNMR rotor



Initial Sample Characterization

2D ^{13}C-^{13}C spectrum



Sensitivity

Resolution

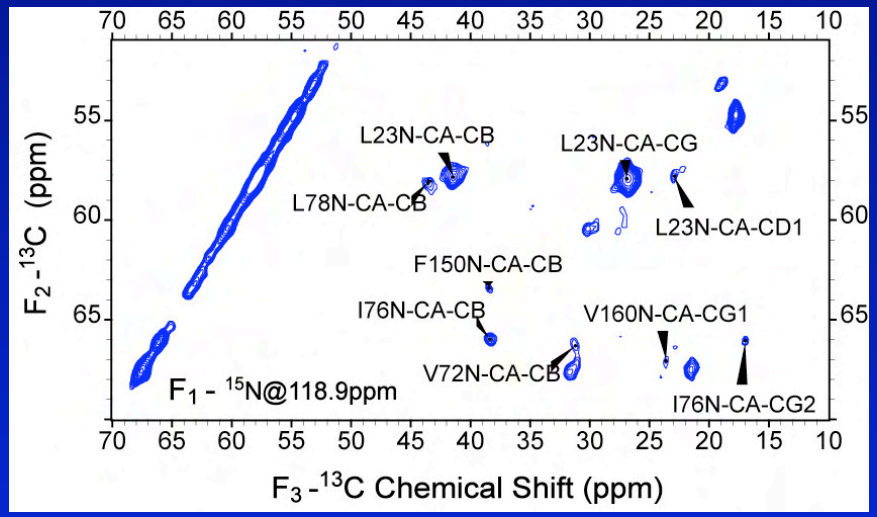
Secondary structure

Reproducibility

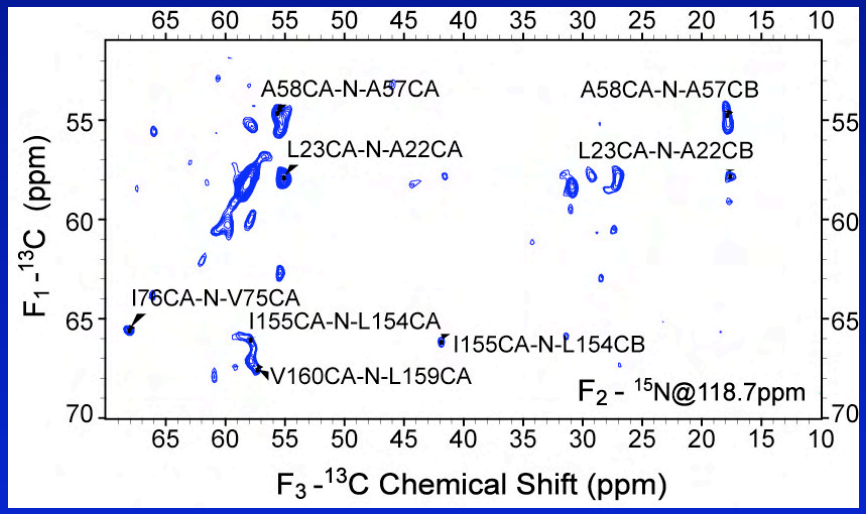
Li, Berthold, Frericks, Gennis & Rienstra, *ChemBioChem* 2007, 8: 434-442

3D Experiments: Sequential Signal Assignments

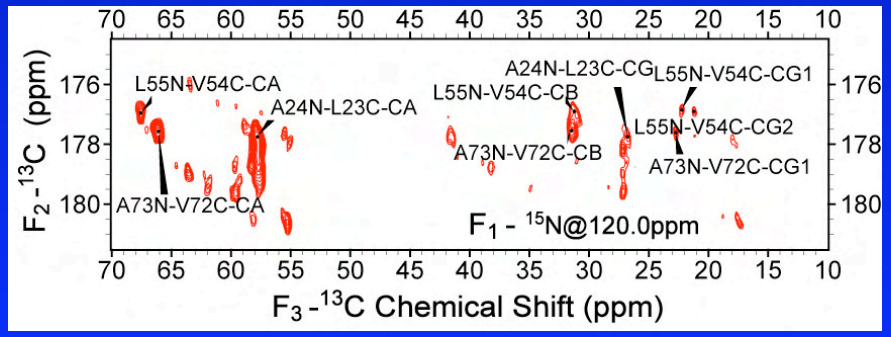
NCACX 3D



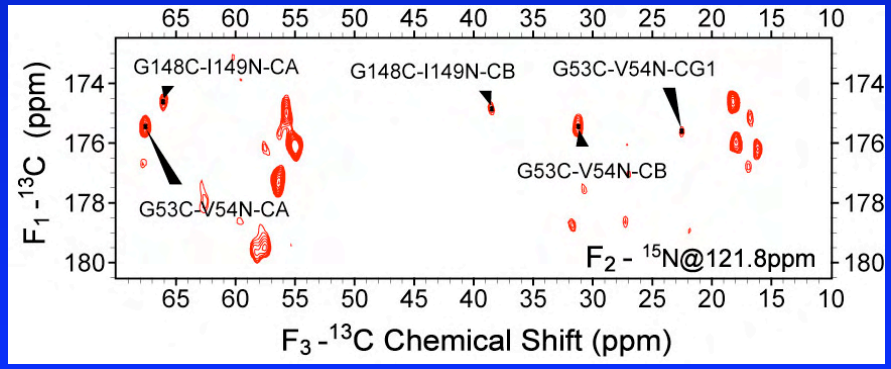
CAN(CO)CX 3D



NCOCX 3D

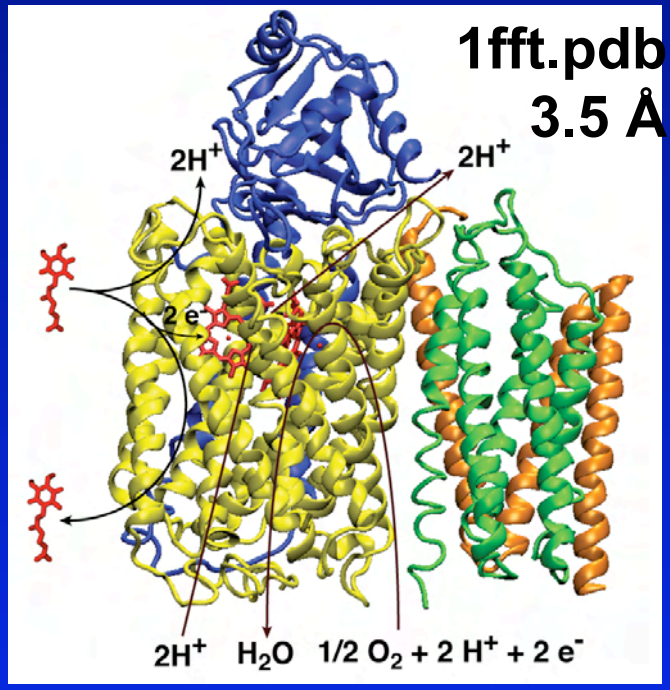


CON(CA)CX 3D



Cytochrome bo_3 Oxidase

Integral Membrane Protein
144 kDa, 1,291 residues

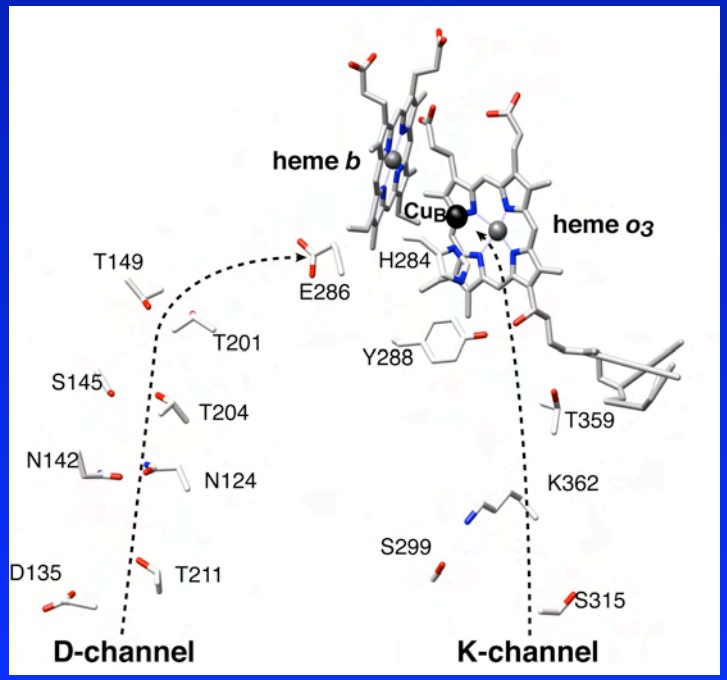


Robert Gennis

Heather Fricks
Lai Lai Yap
Myat Lin

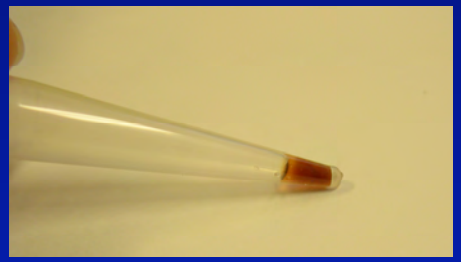
Why SSNMR? Unknown Mechanistic Information

- Interactions in quinol binding sites
- Gating of H^+ Channels
- Protonation states
- Dynamics

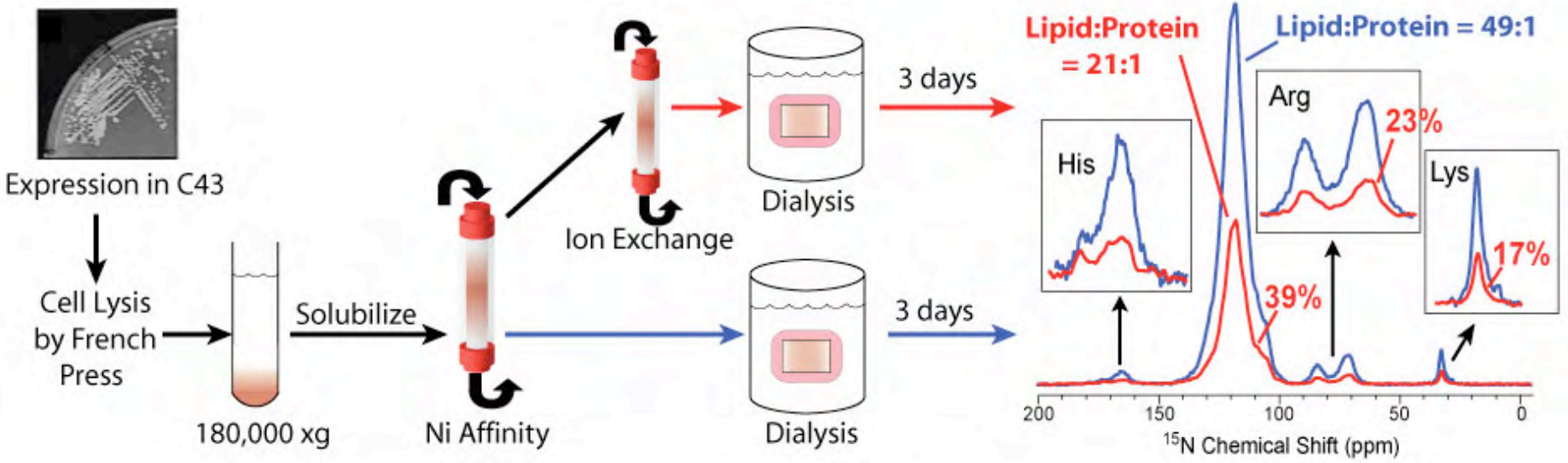


Cytochrome bo_3 Preparation

- Expression in E. *coli* C43
- Minimal media
  - 2 g/L ^{13}C glycerol
  - 2 g/L ^{15}N ammonium chloride
- Induction by IPTG
- ~5-6 mg/L overall yield

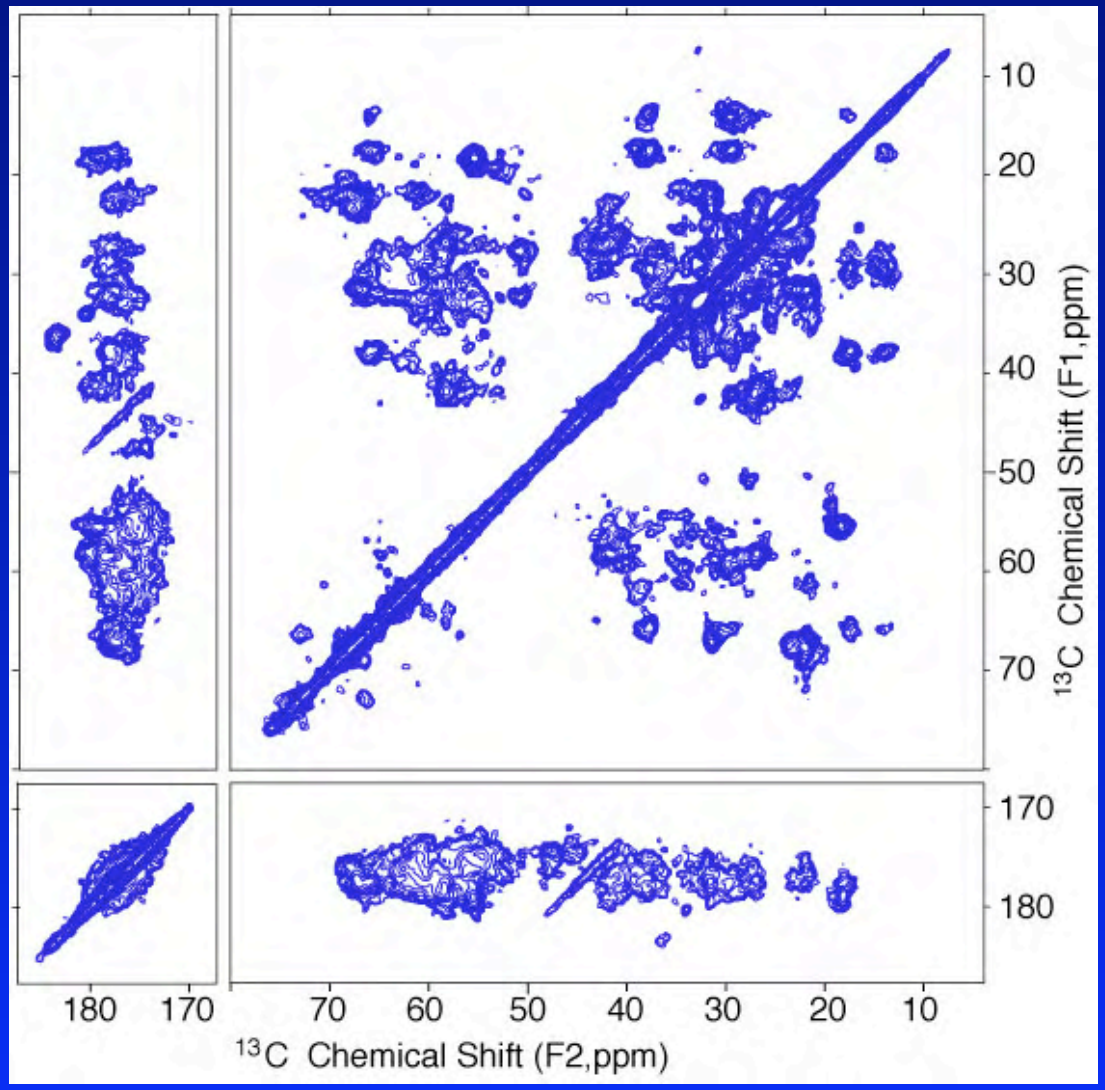


Heather Fericks



Frericks, Zhou, Yap, Gennis & Rienstra, *J. Biomol. NMR* 36:55 (2006).

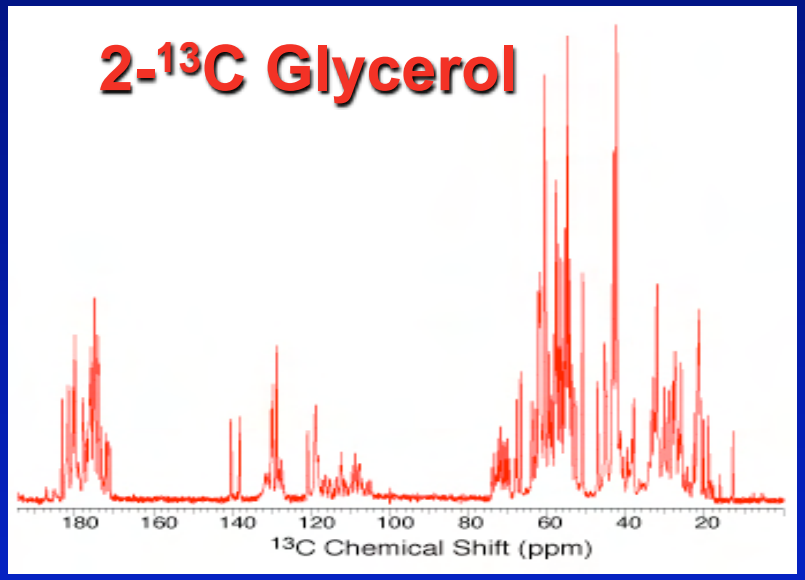
2D ^{13}C-^{13}C Spectrum

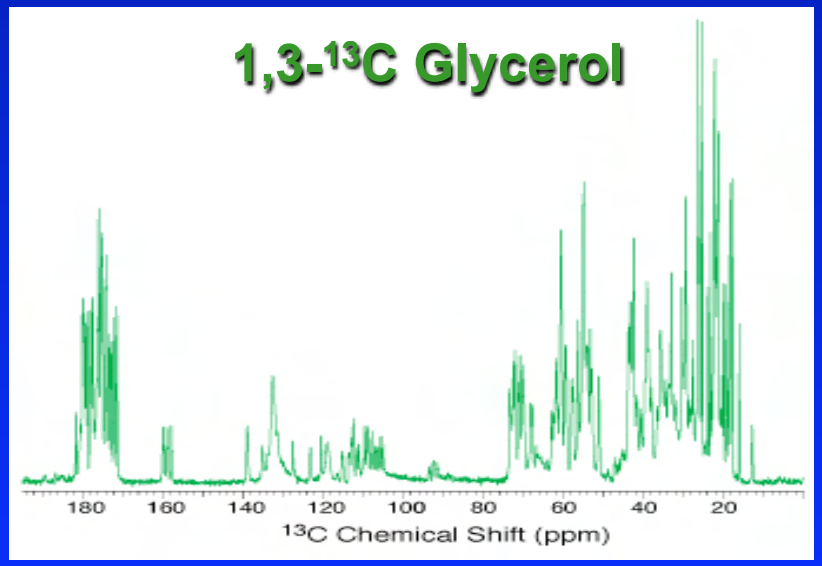


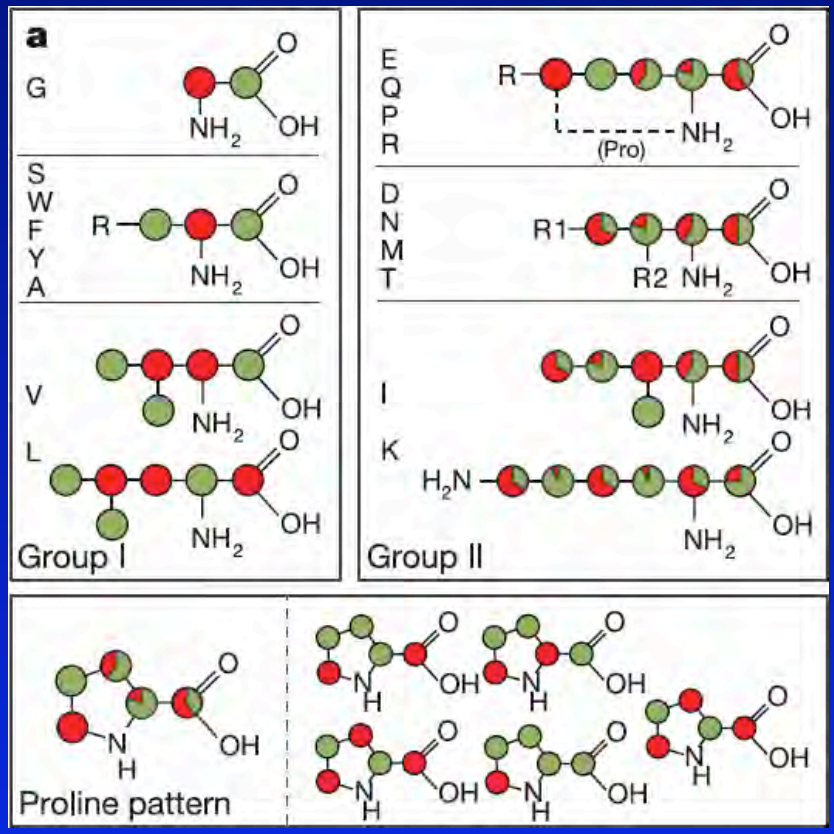
- 100 nmol U-$^{13}C^{15}N$ Cytochrome bo_3
- Line widths < 0.5 ppm
- 16 hours
- 750 MHz
- Varian Bio-MAS Probe

Stringer, Bronnimann, Mullen, Zhou, Stellfox, Li, Williams & Rienstra, *J. Magn. Reson.* (2005).

Glycerol Labeling Scheme







Castellani & Oschkinat, *Nature* 420: 98 (2002)
LeMaster & Kushlan, *J. Am. Chem. Soc.* 118:9255 (1996)

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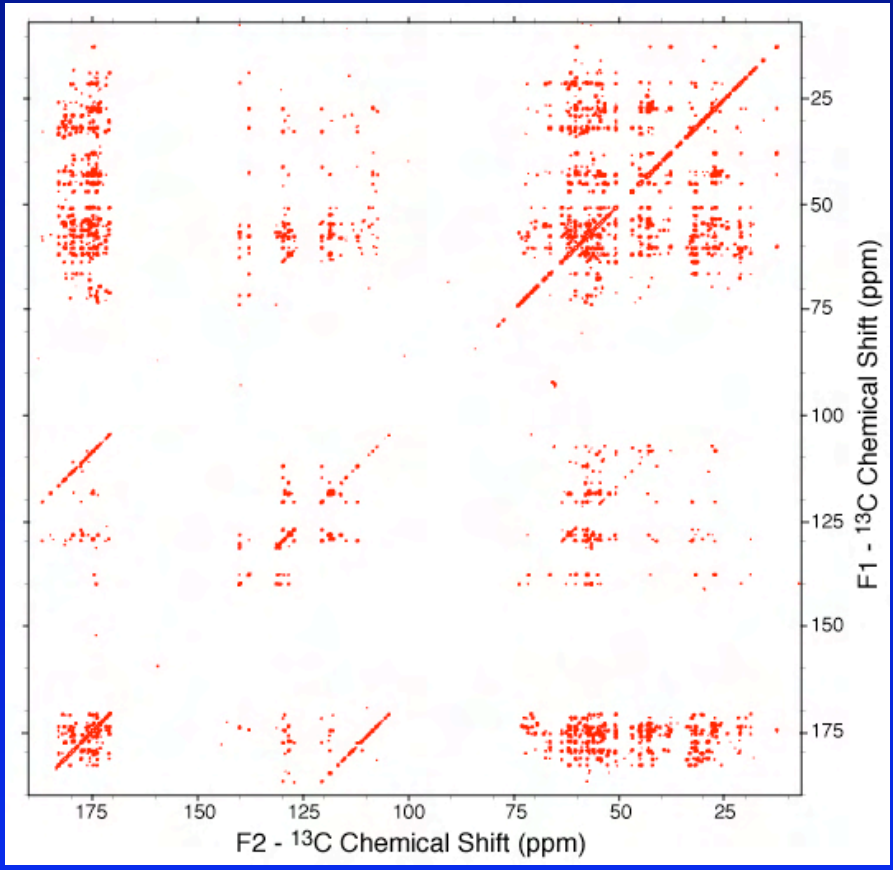
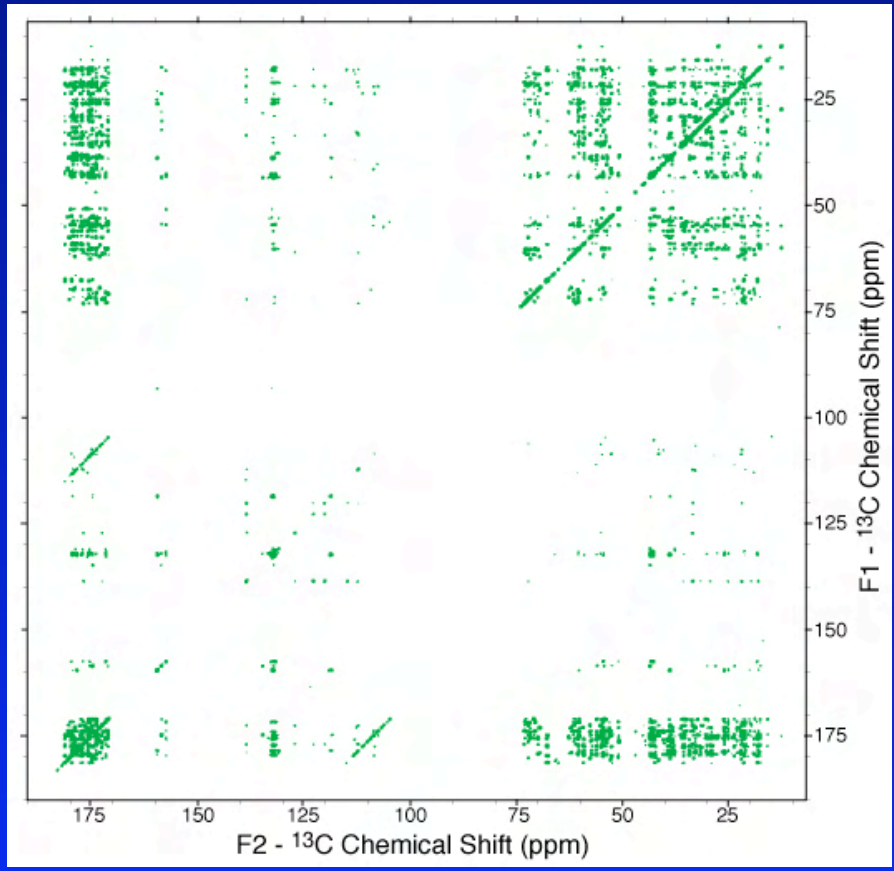
2D 750 MHz Spectra, GB1 (Glycerol)

2-^{13}C-Glycerol
500 ms DARR



1,3-^{13}C-Glycerol
500 ms DARR



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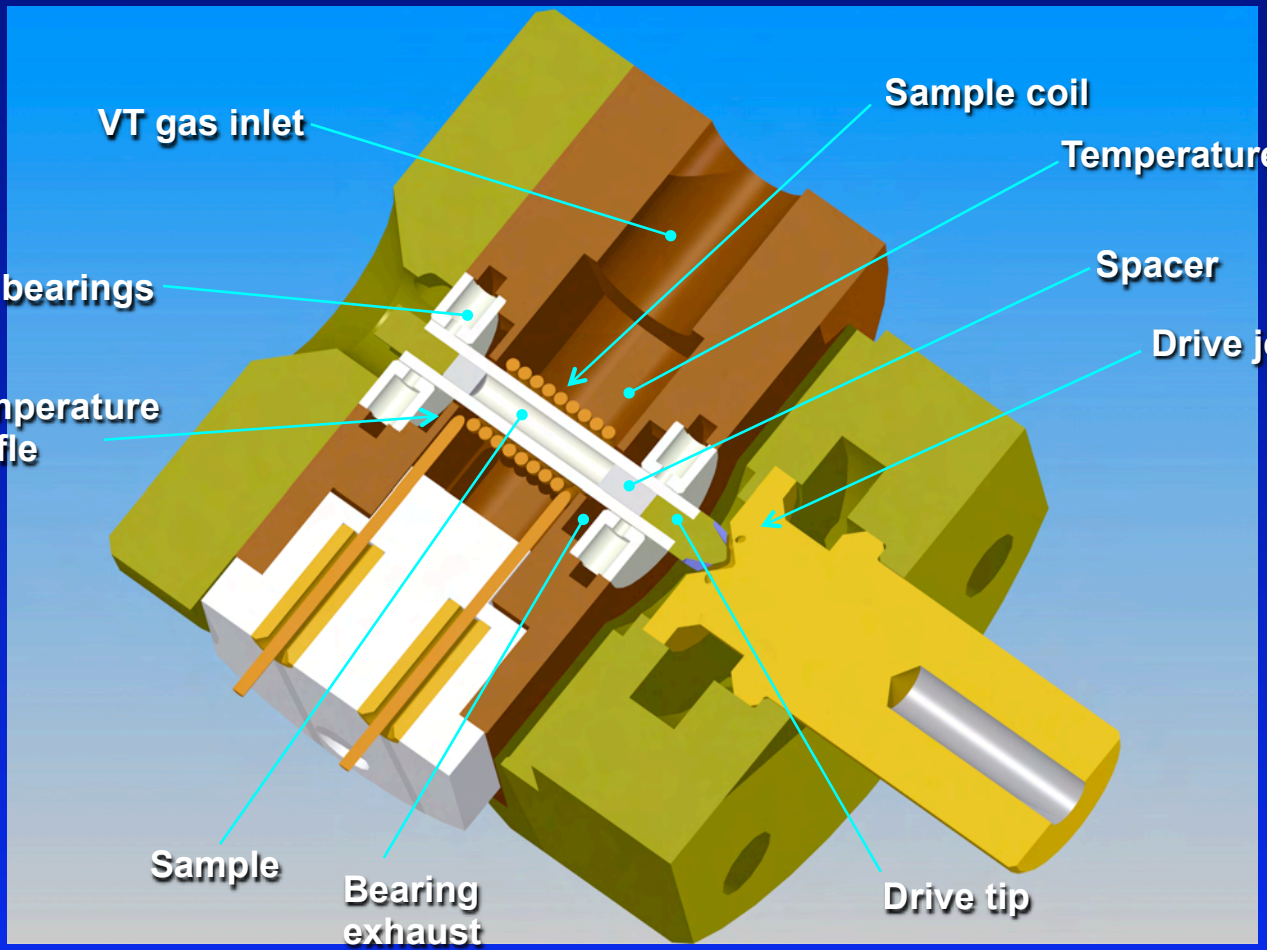
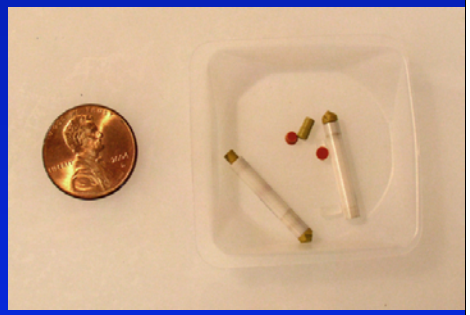
Varian Fast™ & UltraFast™ Spinner

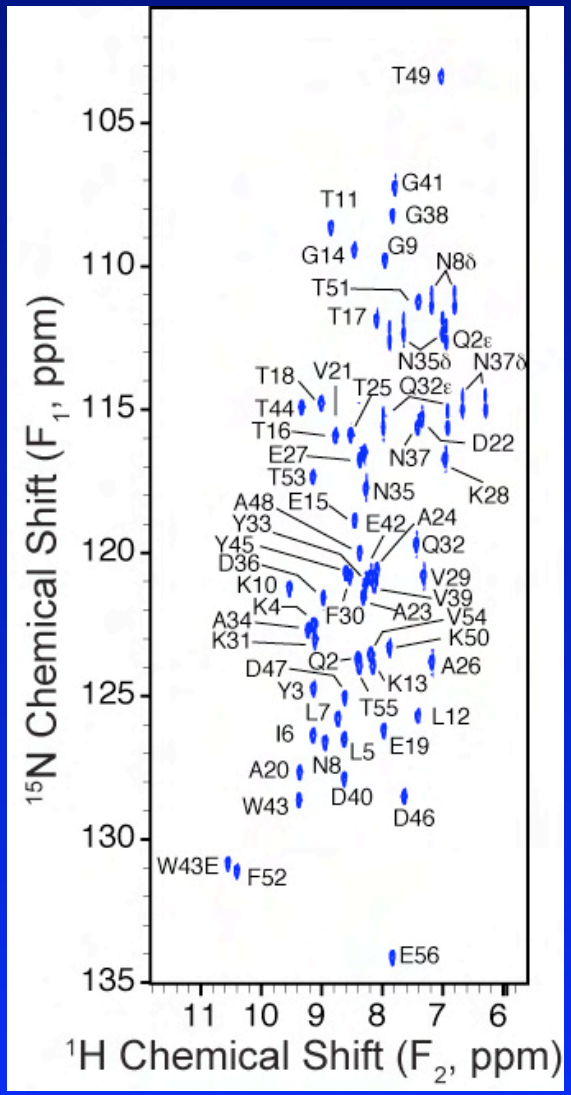




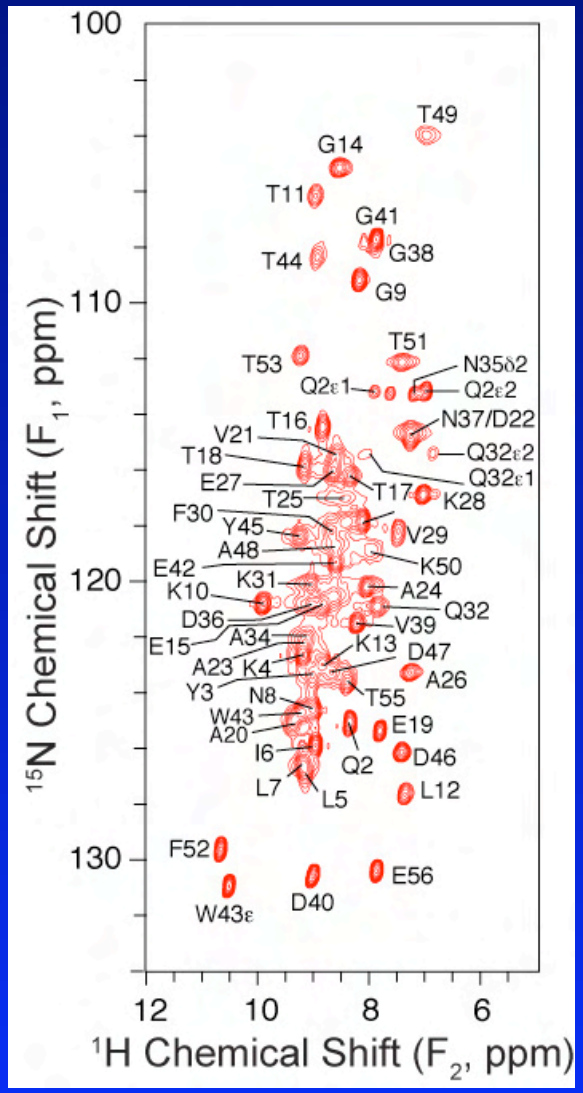
FastMAS: 1.6 mm, 8 uL, 45 kHz

UltraFastMAS: 1.2 mm, 1.2 uL, 65 kHz

Resolved Proton SSNMR Signals



Solution NH 2D



SSNMR NH 2D

1.6 mm, 8 μL

GB1
Both: ~5 mg
1 μmol

Proton NMR in Solids

^{1}H vs X detection enhancement: $\xi \propto (\Delta_X / \Delta_H)^{1/2}$

CRAMPS

Combined Rotation And Multiple-Pulse Spectroscopy

Fast MAS

Peptides, 30 kHz, ξ=2-3

(Ishii & Tycko, *JACS* 123, 2921 (2001))

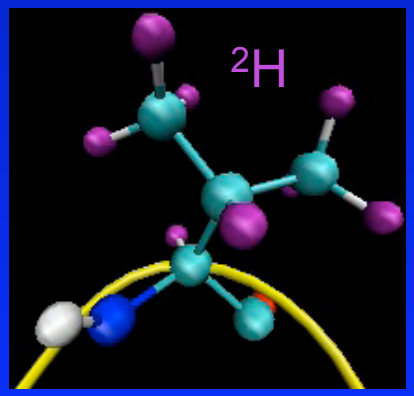
Proton dilution — perdeuteration

Exchange with H_2O,136-312 Hz

(Paulson & Zilm, *JACS* 125, 15831 (2003))

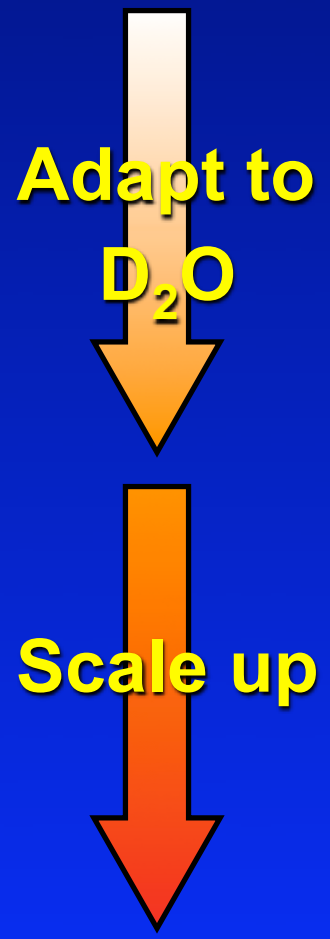
Exchange with 10% H_2O : 90% D_2O, 17-35 Hz

(Chevelkov & Reif, *Angew. Chemie* 128:12620 (2006))



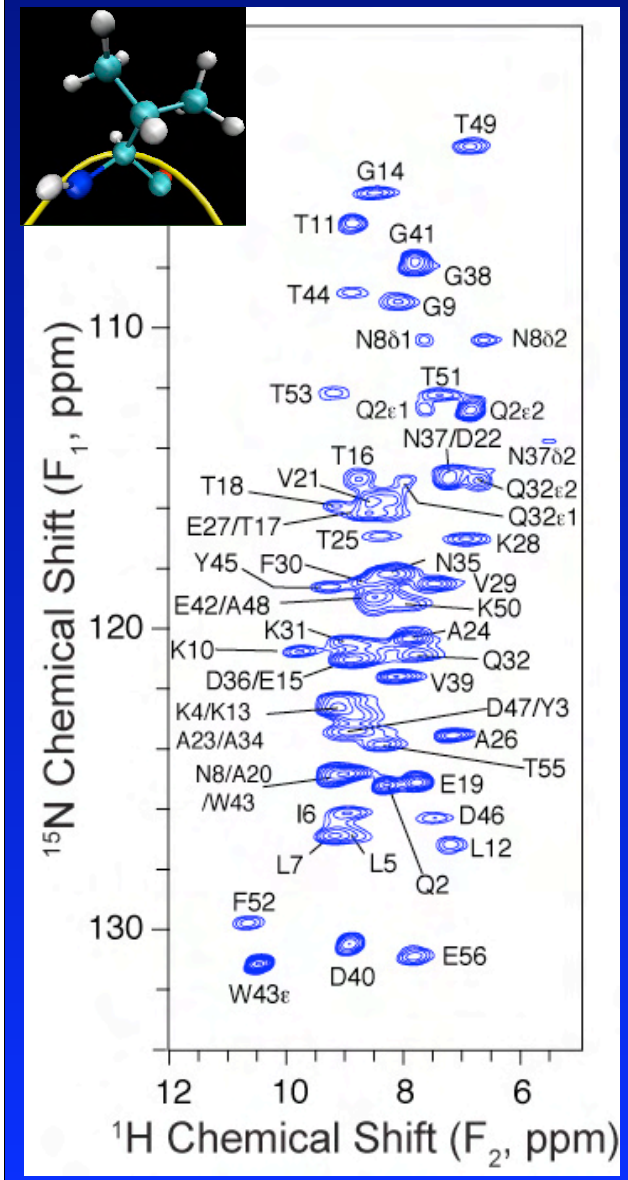
Deuteration for SSNMR

Preculture 0: Grow overnight culture in LB
Preculture 1: Inoculate 2 ml minimal medium w/ 0.1 vol of Preculture 0.
Grow overnight
Preculture 2: Inoculate 2 ml ^{13}C ^{15}N ^{2}D (CND) medium
with 0.1 vol of Preculture 1. (= **90% D_2O**)
Grow 8 hrs.
Preculture 3: Inoculate 2 ml CND medium
with 0.1 vol of Preculture 2 (= **99% D_2O**)
Grow overnight.
Preculture 4: Inoculate 2 ml CND medium
with 0.1 vol Preculture 3 (= **99+% D_2O**)
Grow 8 hr.
Preculture 5: Inoculate 25 ml CND medium
with 0.03 vol Preculture 4
Grow overnight.
Culture: Inoculate 1 L CND medium
with 0.03 vol Preculture 5
Grow 8+ hr to A600 = 0.6
Induce with 0.5 mM IPTG
Harvest at 10+ hr



Cai et al. (1998)
J. Biomol. NMR 11, 97

The Importance of Being Deuterated



← 100% ^{1}H

Δ_H **360** ± 115 Hz

s/n **185** ± 77

ξ **14** ± 3

Deuterated →

Δ_H **149** ± 40 Hz

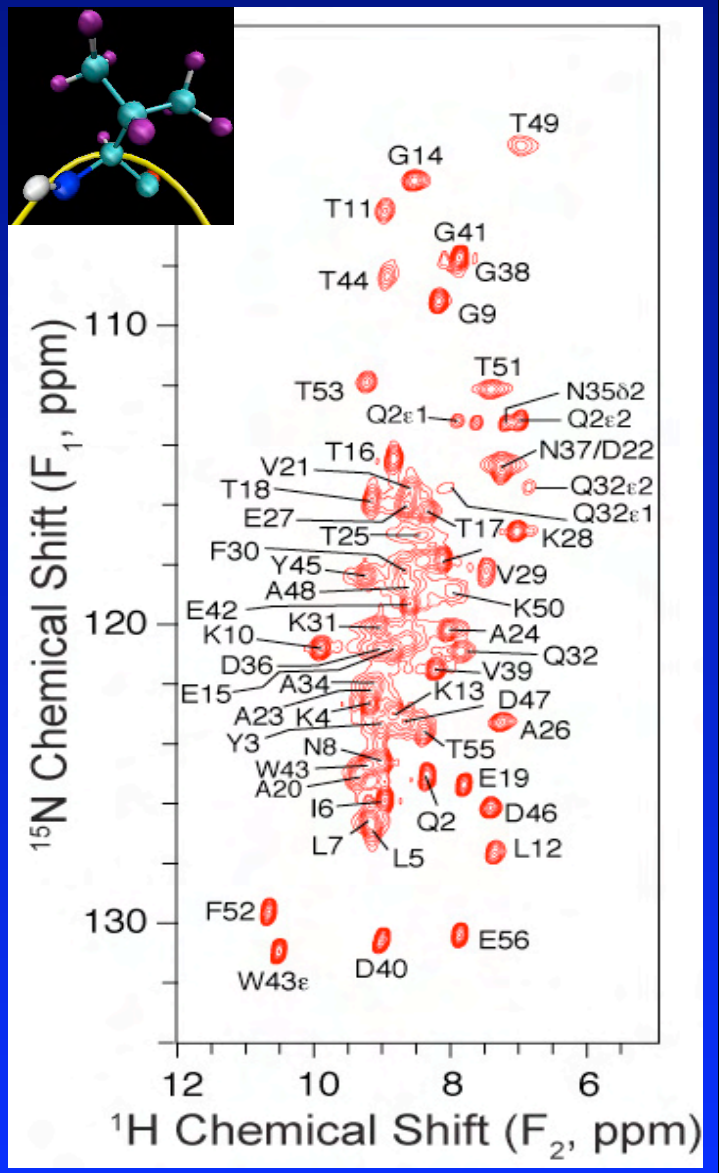
T_2 7 ms => ~ 50 Hz

shimming ~ 60 Hz

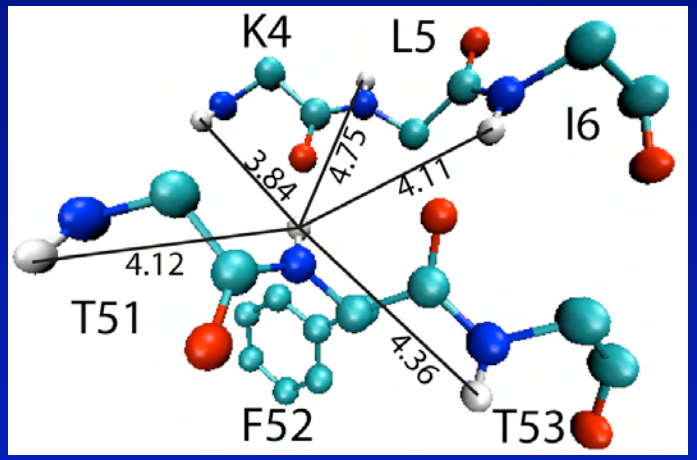
s/n **457** ± 187

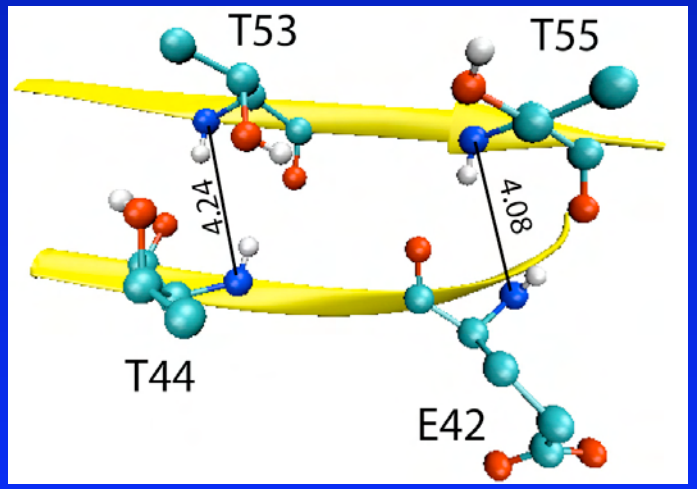
ξ **18** ± 3

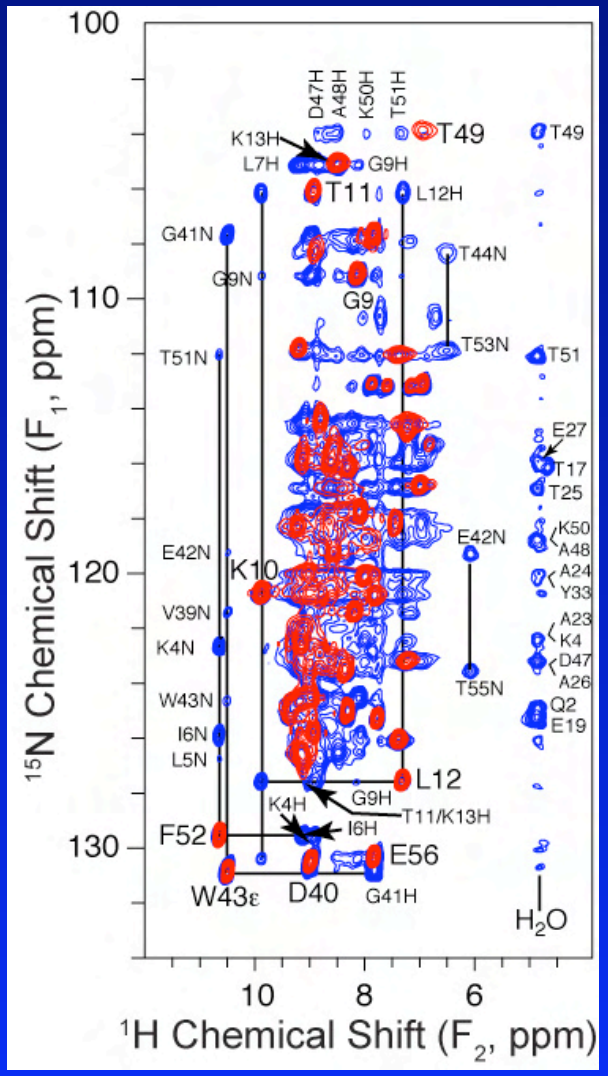
Both
30 min, 2 scans, 750 MHz



Resolved Distance Restraints







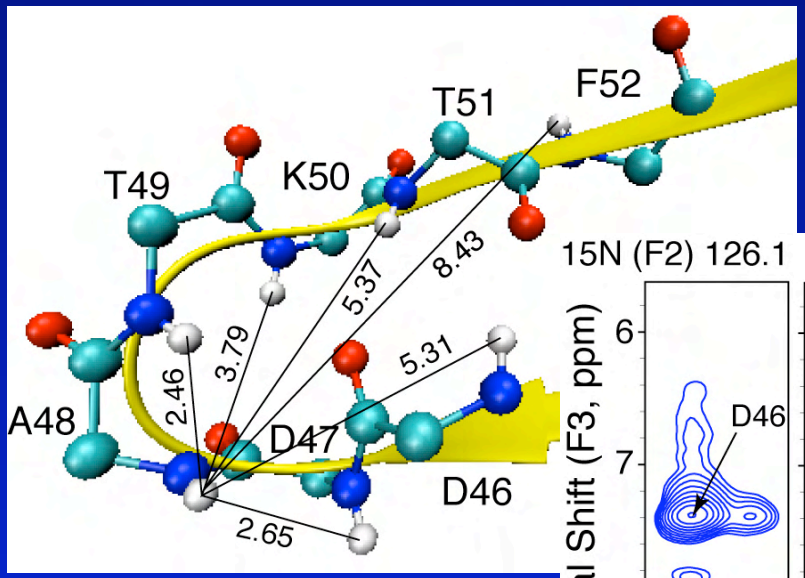
Dr. Donghua Zhou

Scroll resonator (BioMAS™)

Inova code (Solids BioPack™)

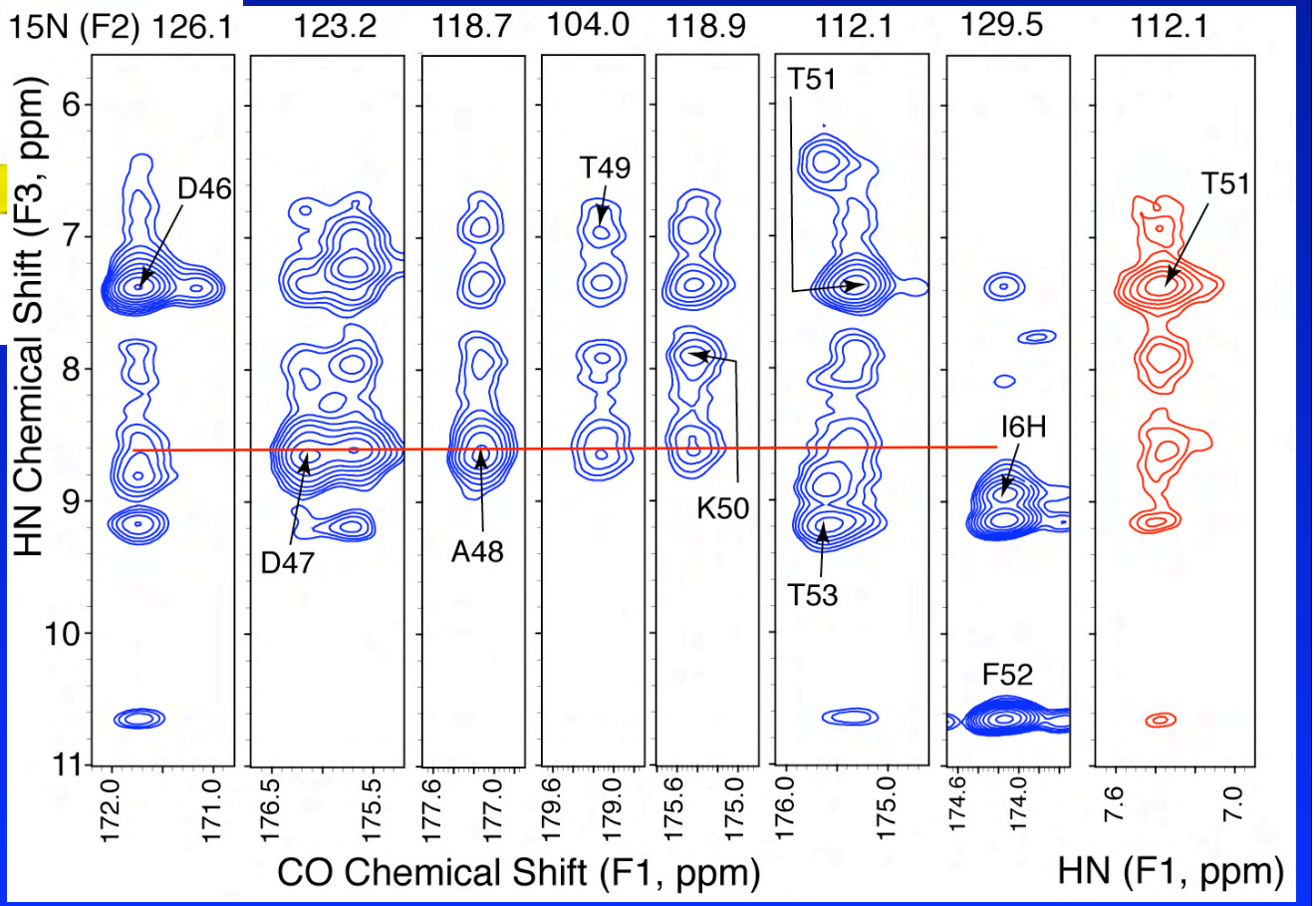
1.6 mm, 45 kHz (FastMAS™)

3D Experiments Limit Degeneracies



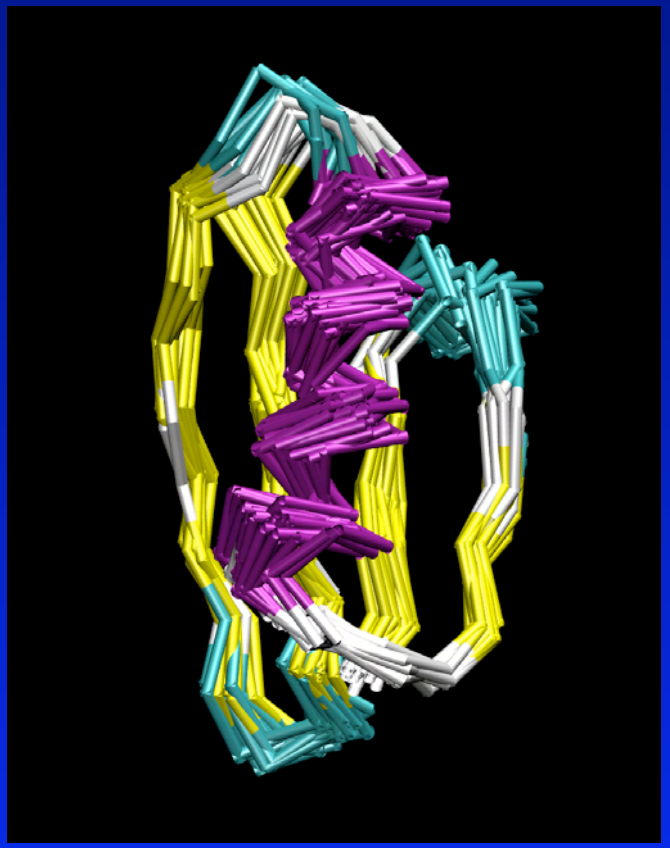
2 ms RFDR ^{1}H-^{1}H mixing

CON(H)H HN(H)H



HN(H)H: Paulson & Zilm
***JACS* 125:14222 (2003)**

Structure of GB1 from Proton Distances



- 154 ^{1}H-^{1}H distance restraints
- Iterative assignment
- TALOS dihedral restraints
- Standard XPLOR-NIH calculation
- Family of 20 structures from 500
- 1.1 Å backbone RMSD

Rienstra Group
Experiment Configuration

Samples Required:

80% Adamantane + 20% KBr

^{13}C,^{15}N-N-acetyl-valine or other peptide

A larger protein (preferably GB1)

Goals

Basic instrument setup

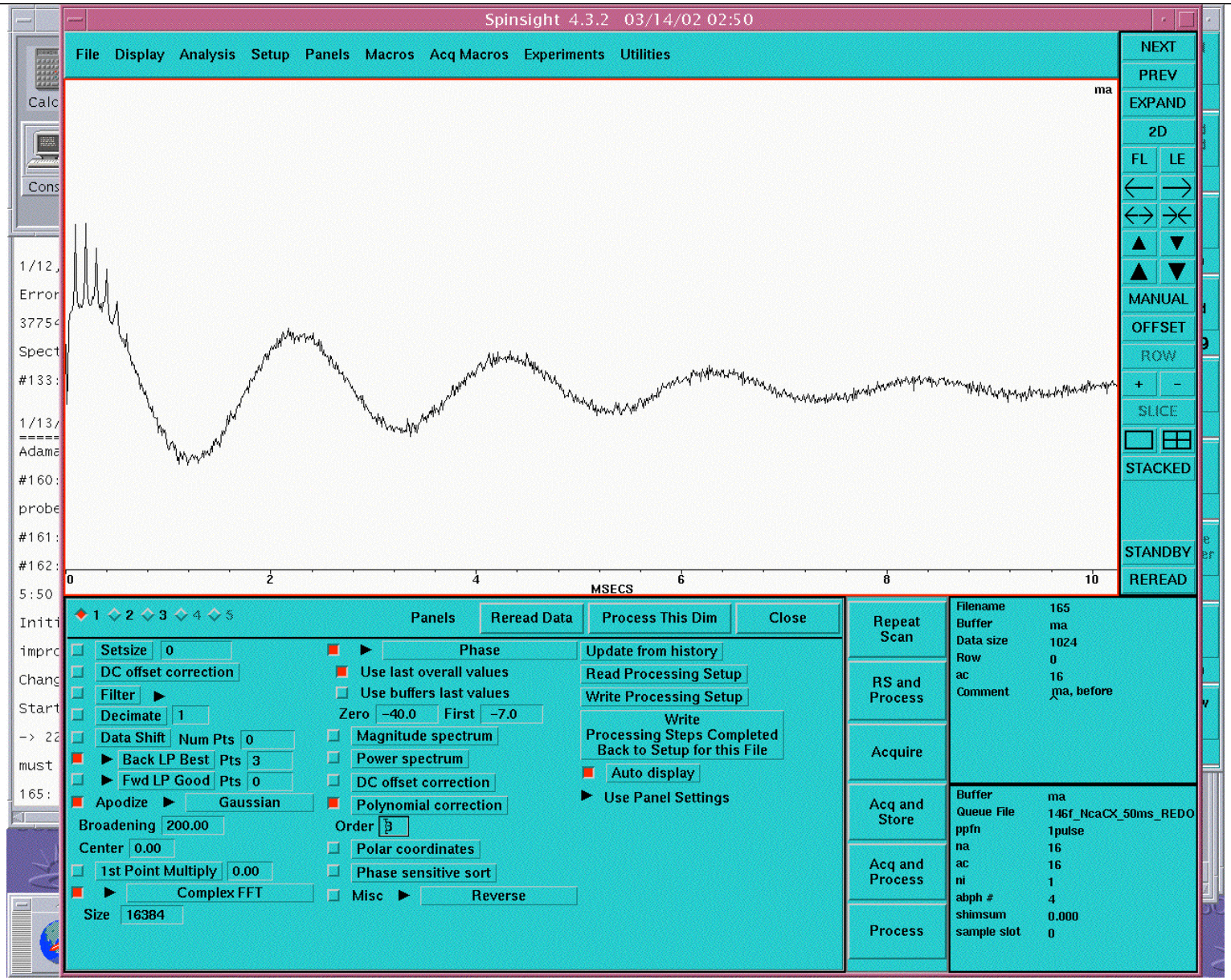
Set pulse widths and easy CP condition

Optimize finicky CP conditions & decoupling

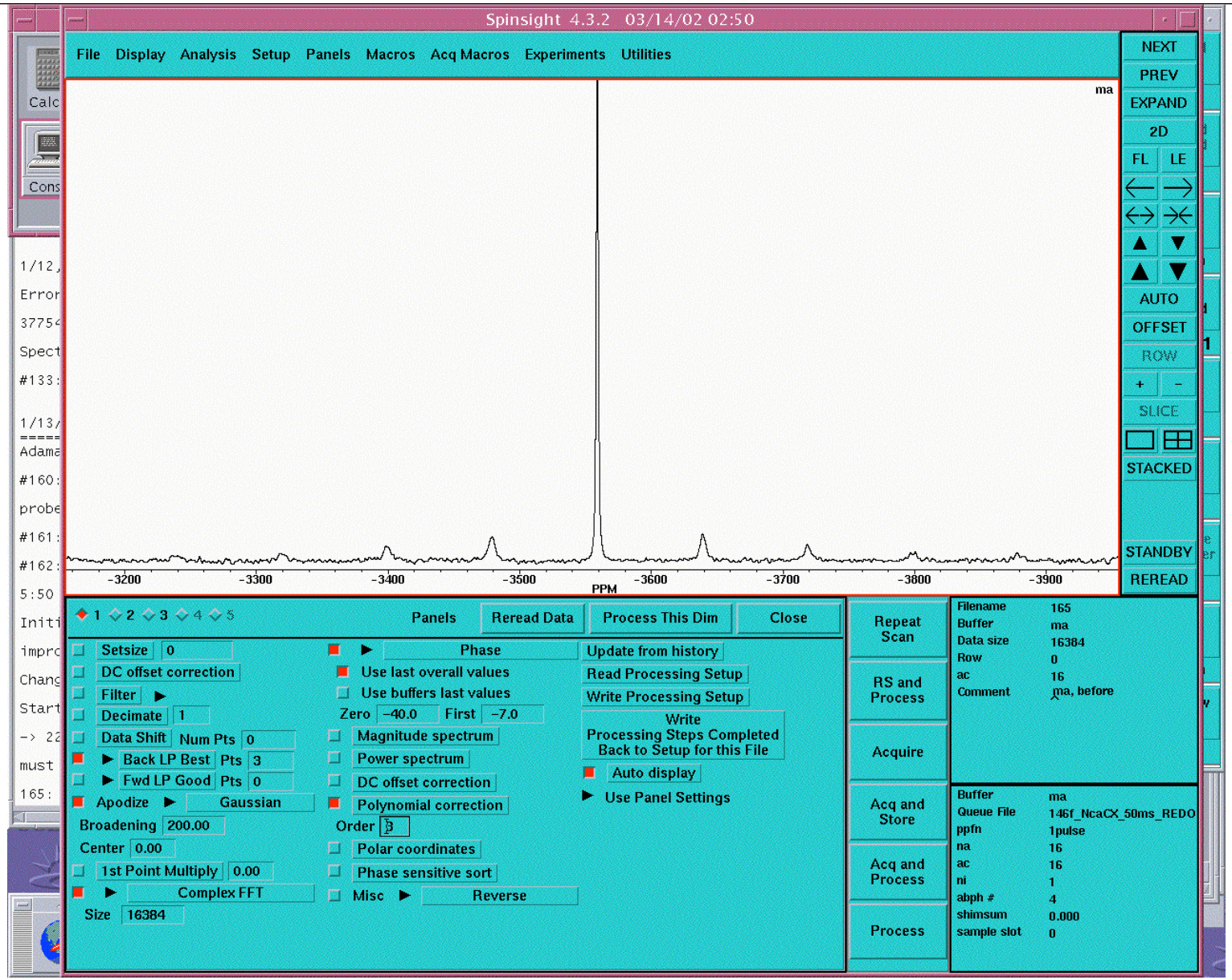
Part One:
Setting the Magic-Angle & Shimming

Sample:
80% Adamantane + 20% KBr
(physical mixture)

Anticipated Time Requirement
Initial setup: 2-3 hours
Confirming earlier setup: 20-30 minutes

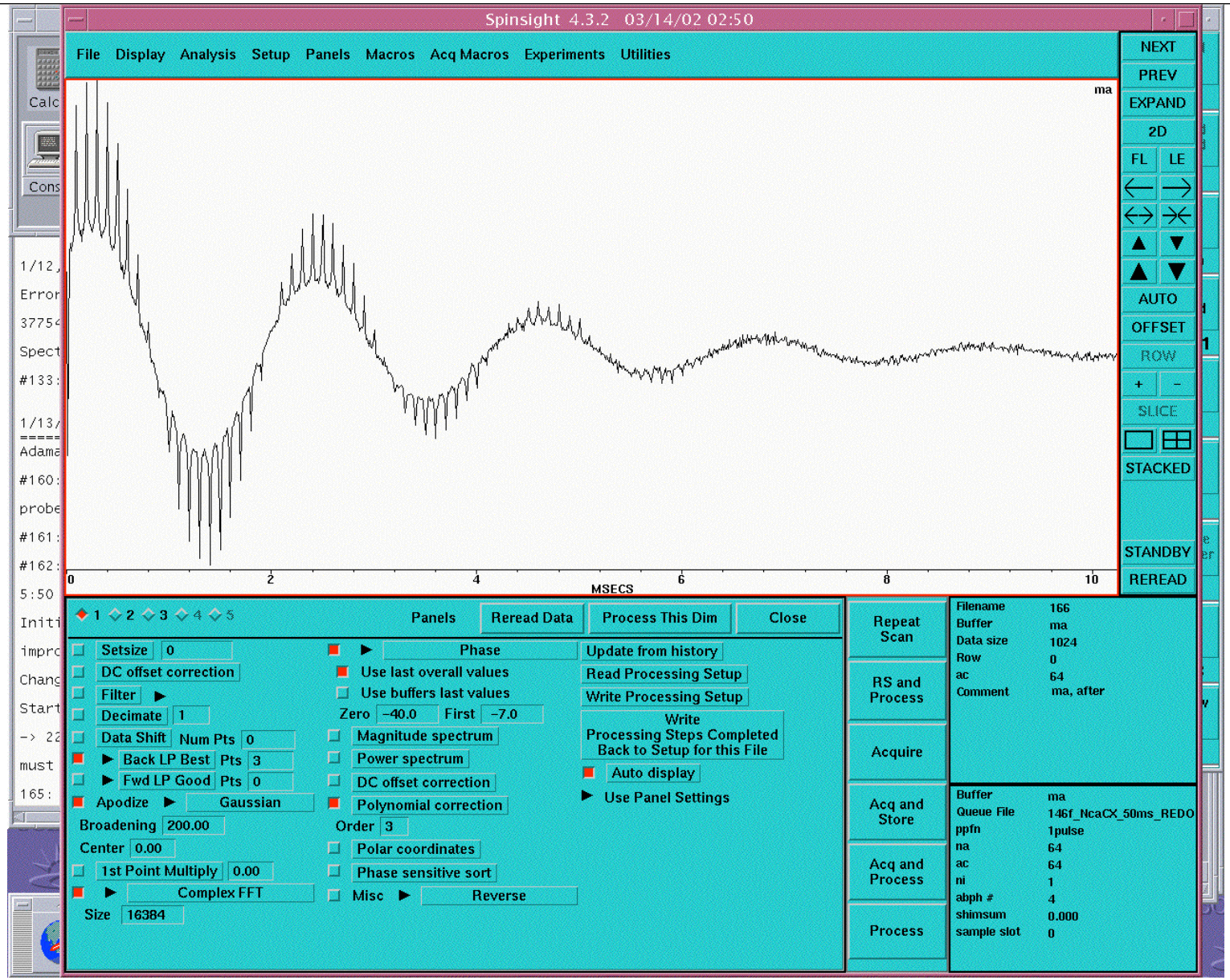


This screen illustrates a poorly set magic angle in the time domain. The rotational echoes extend only for about 1 ms, with from the $K^{79}Br$ signal at 10 kHz MAS (500 MHz ^{1}H frequency). This magic angle is badly in need of adjustment.

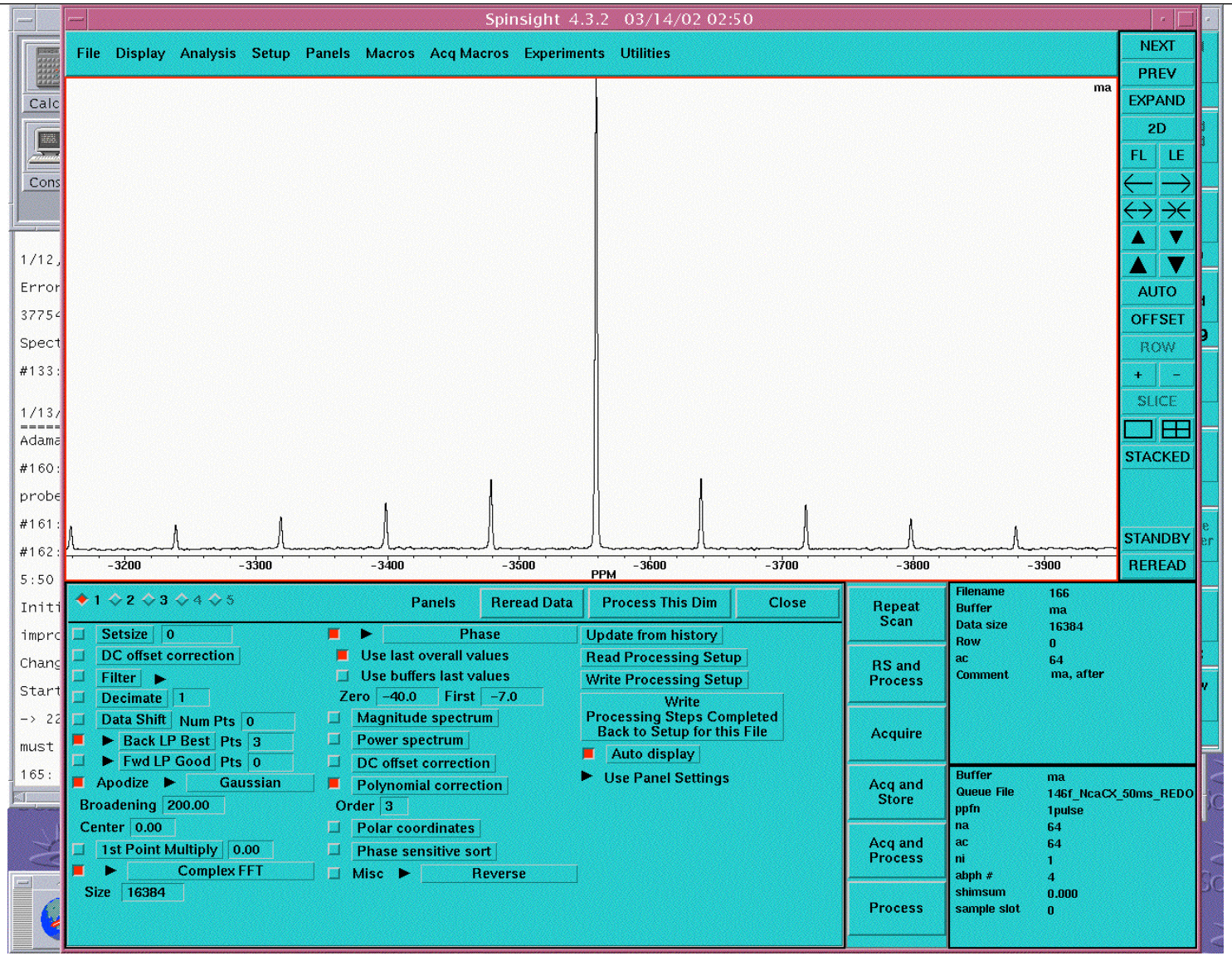


This screen shows the same data processed in the frequency domain.

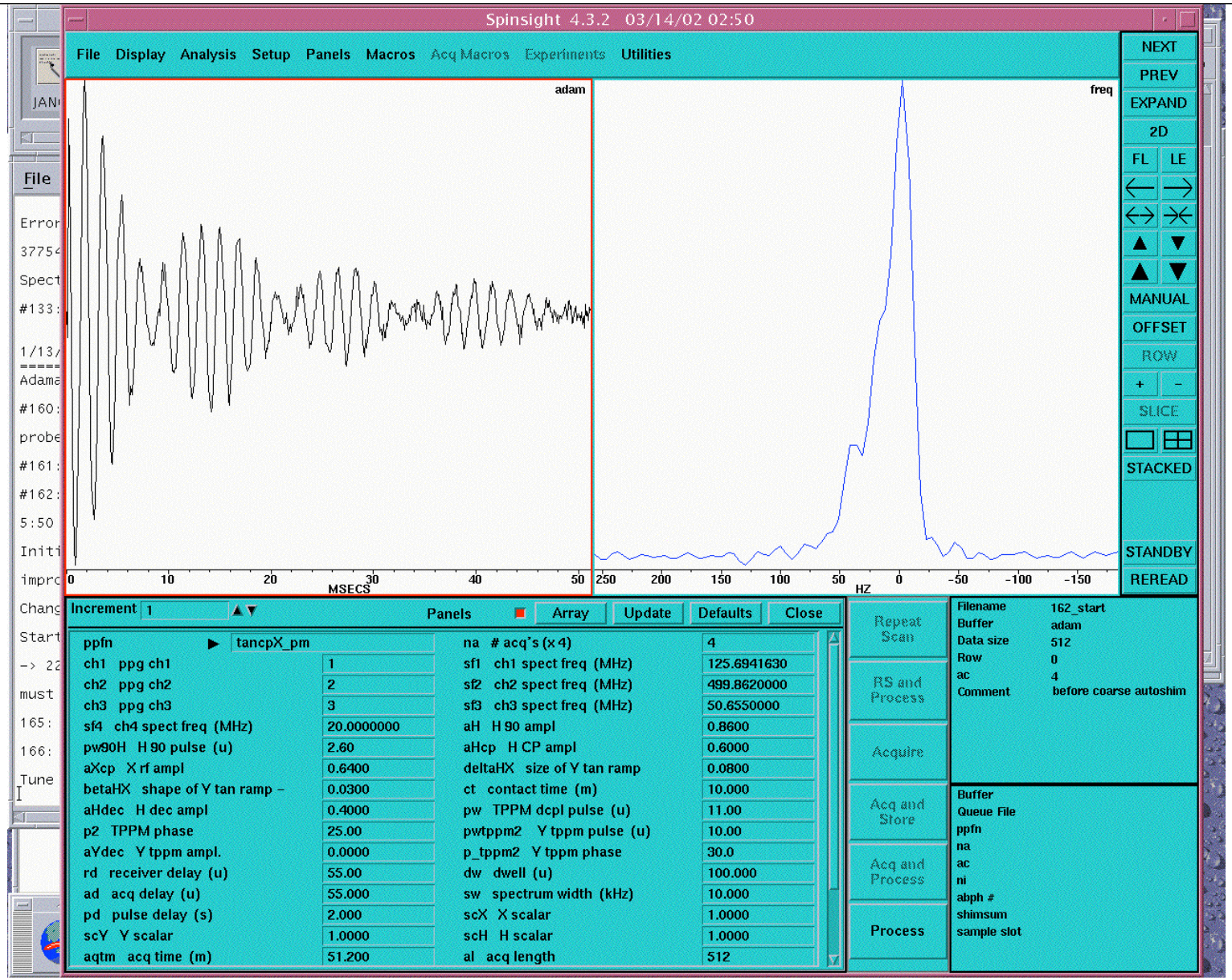
The first order sidebands are broader than the centerband, and only ~5% of its intensity.



This snapshot illustrates a properly set magic angle, under the same conditions of 10 kHz MAS on a 500 MHz instrument. The rotational echoes extend out to more than 6 ms.

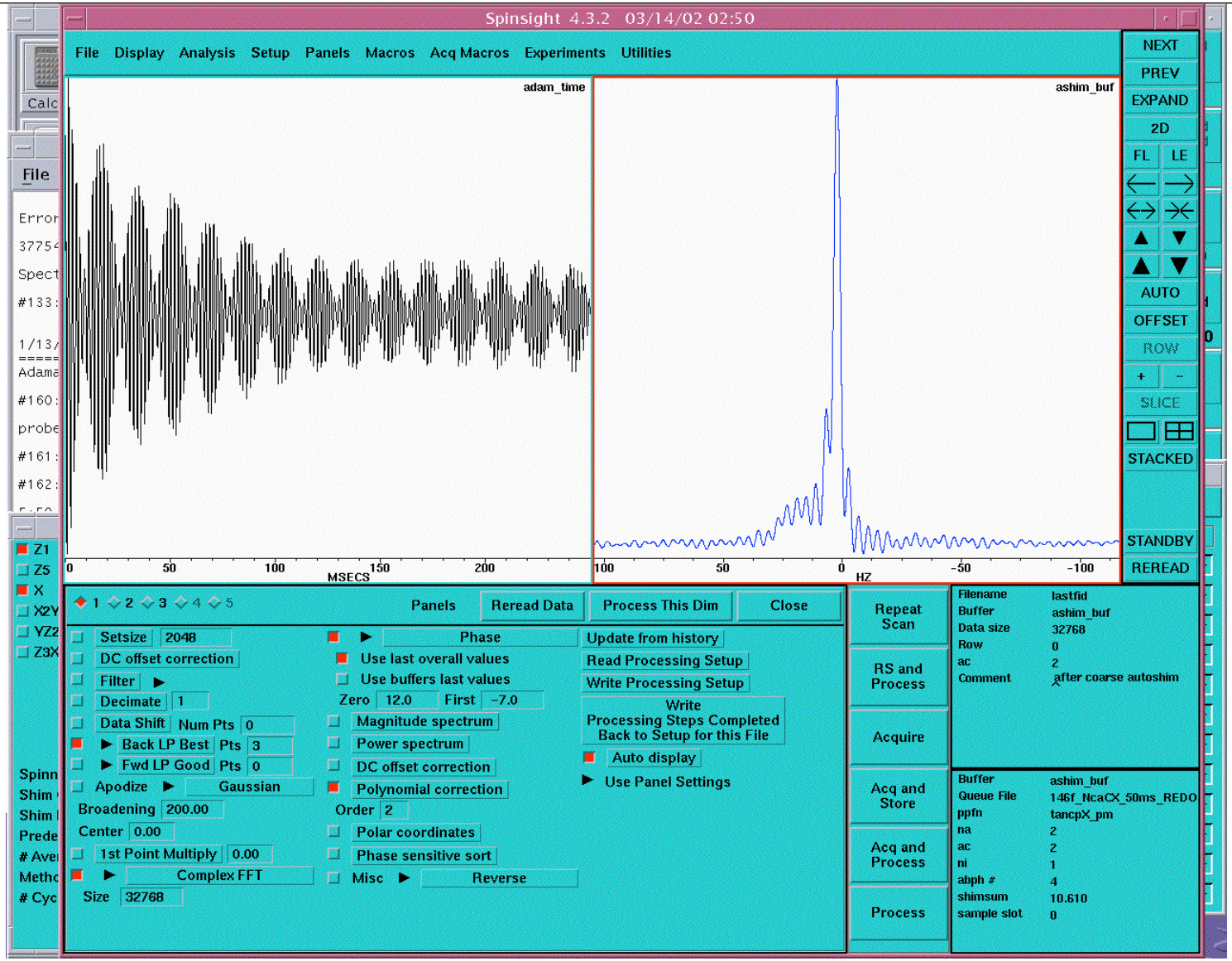


In the frequency domain, the difference is equally evident. The first order sidebands are between 14.5 and 15.0% of the centerband, the second order sidebands are 9.5-10%, and the line widths of the sidebands are all similar to the centerband.



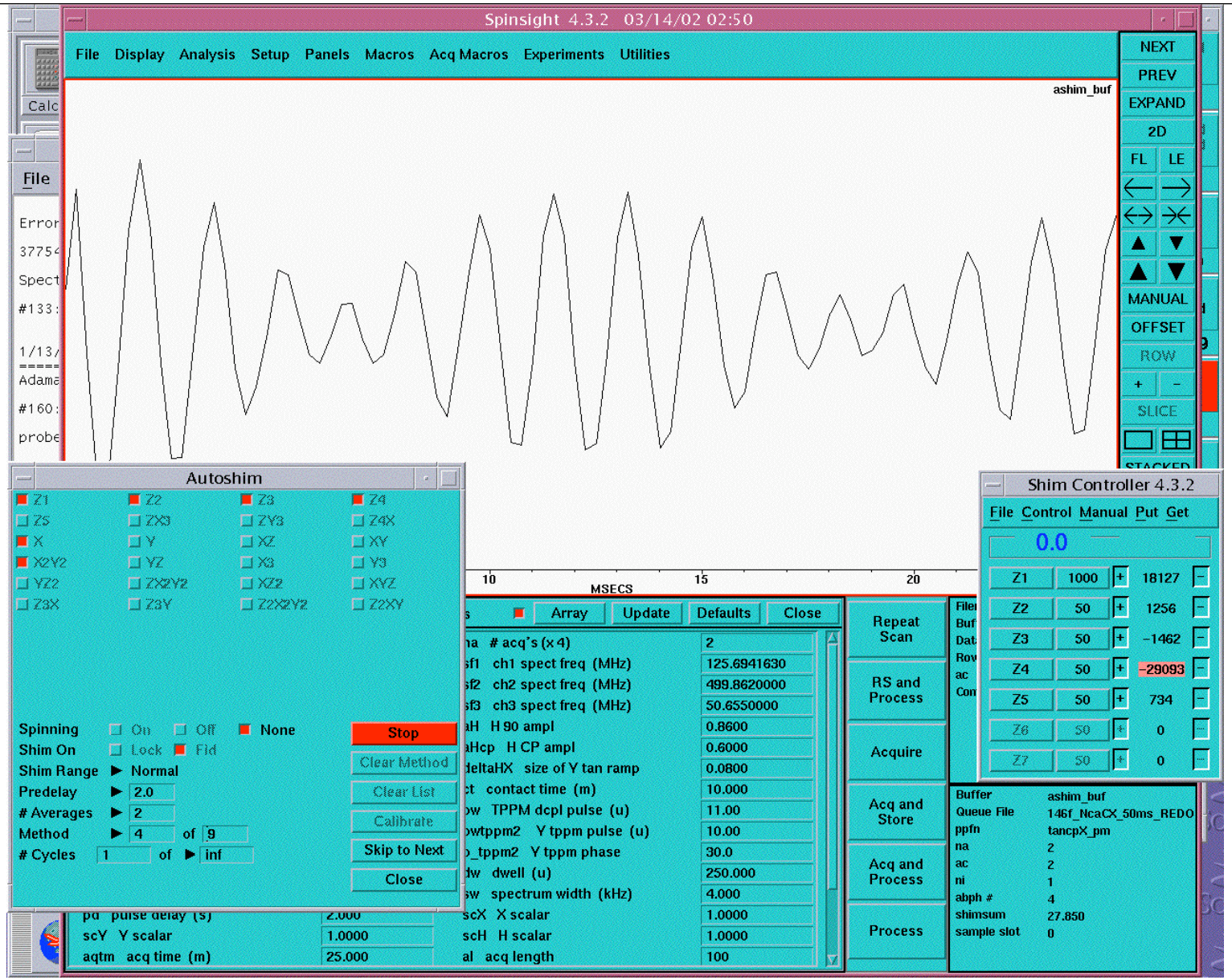
This adamantane signal comes from an unshimmed probe (all the shims are set to zero).

The line shape is reminiscent the early 50's when Herb Gutowsky was in his prime.

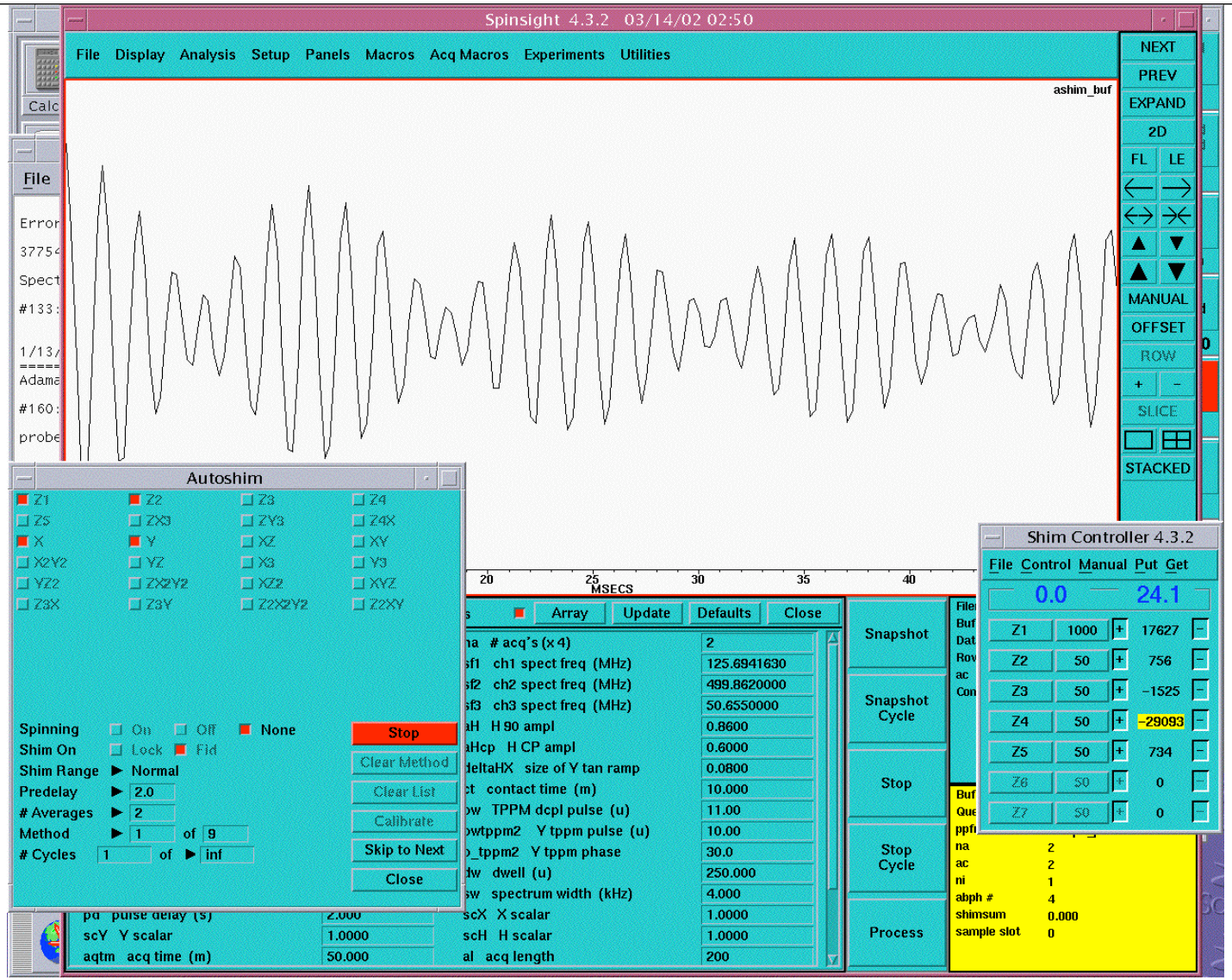


Here is the result from autoshimming only on low order shims: Z1, X, Y, Z2, X2Y2, XZ, YZ.

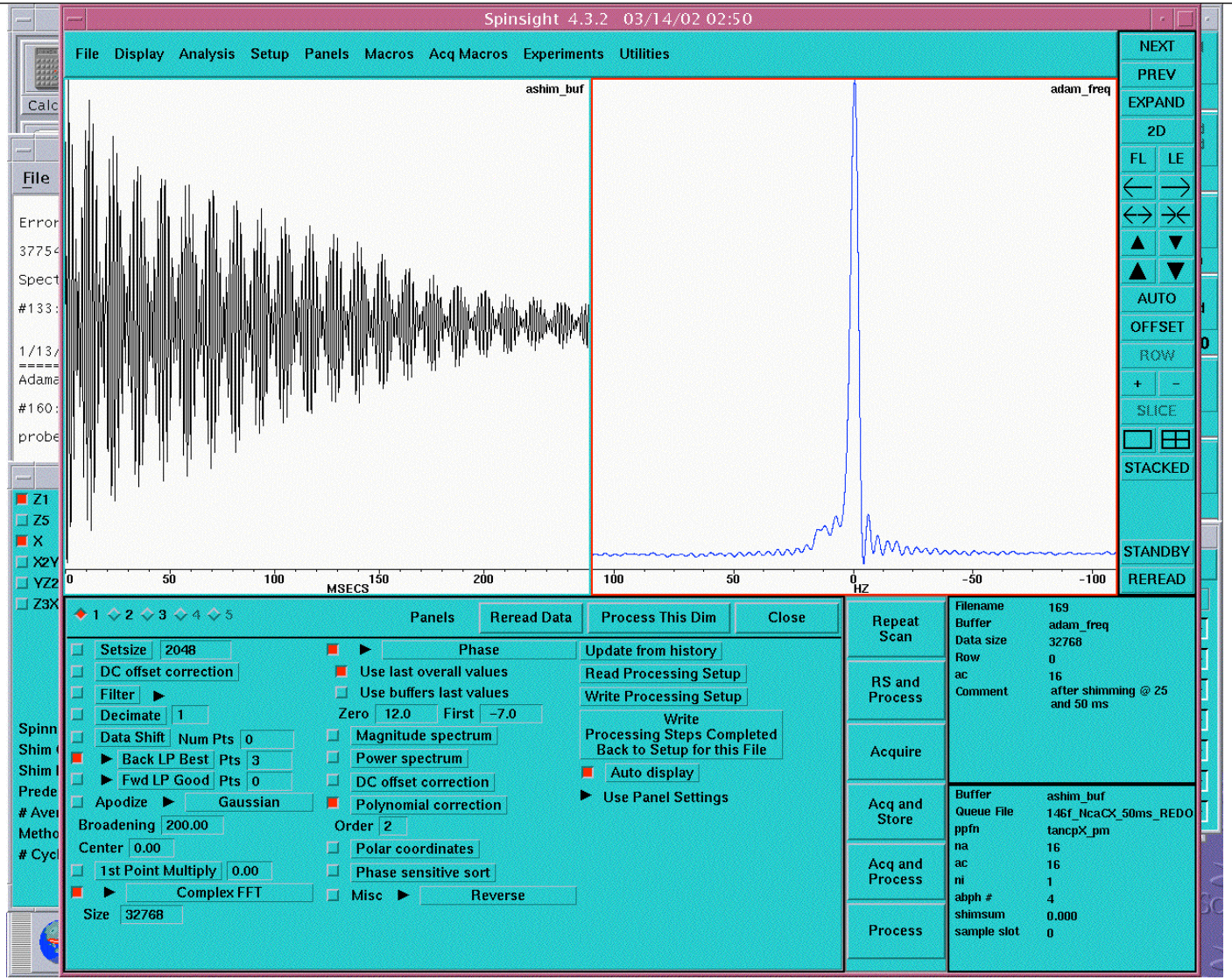
A very narrow component can be obtained, but more than half of the time domain decay occurs in the first 100 ms, consistent with the downfield “foot” in the frequency domain.



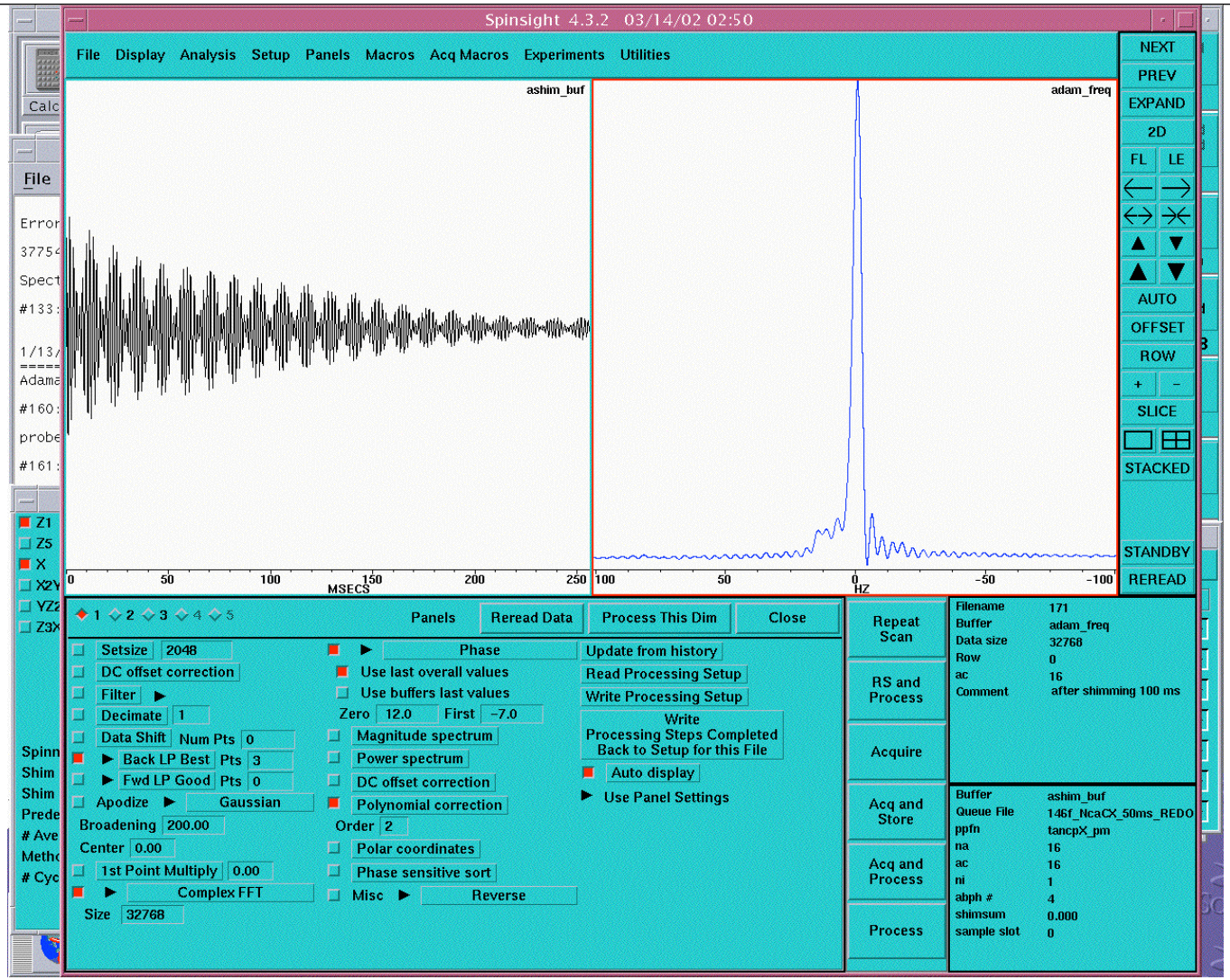
To remove the broad feature from the line shape, go back and autoshim using a standard method file (e.g., autoshim_normal.ashim, which includes 9 methods), but using a time domain window of only ~25 ms. Any improvements derived will be specific to the “foot”.



After the autoshimming routine converges (~30 minutes) with the short time domain window (in this case the shimsum score was ~28), we extend the time window to ~50 ms. If the shimsum stays constant as longer times, you have perfect shimming; the decrease to ~23 indicates that there is room for improvement. After more work, it comes back up to ~25.



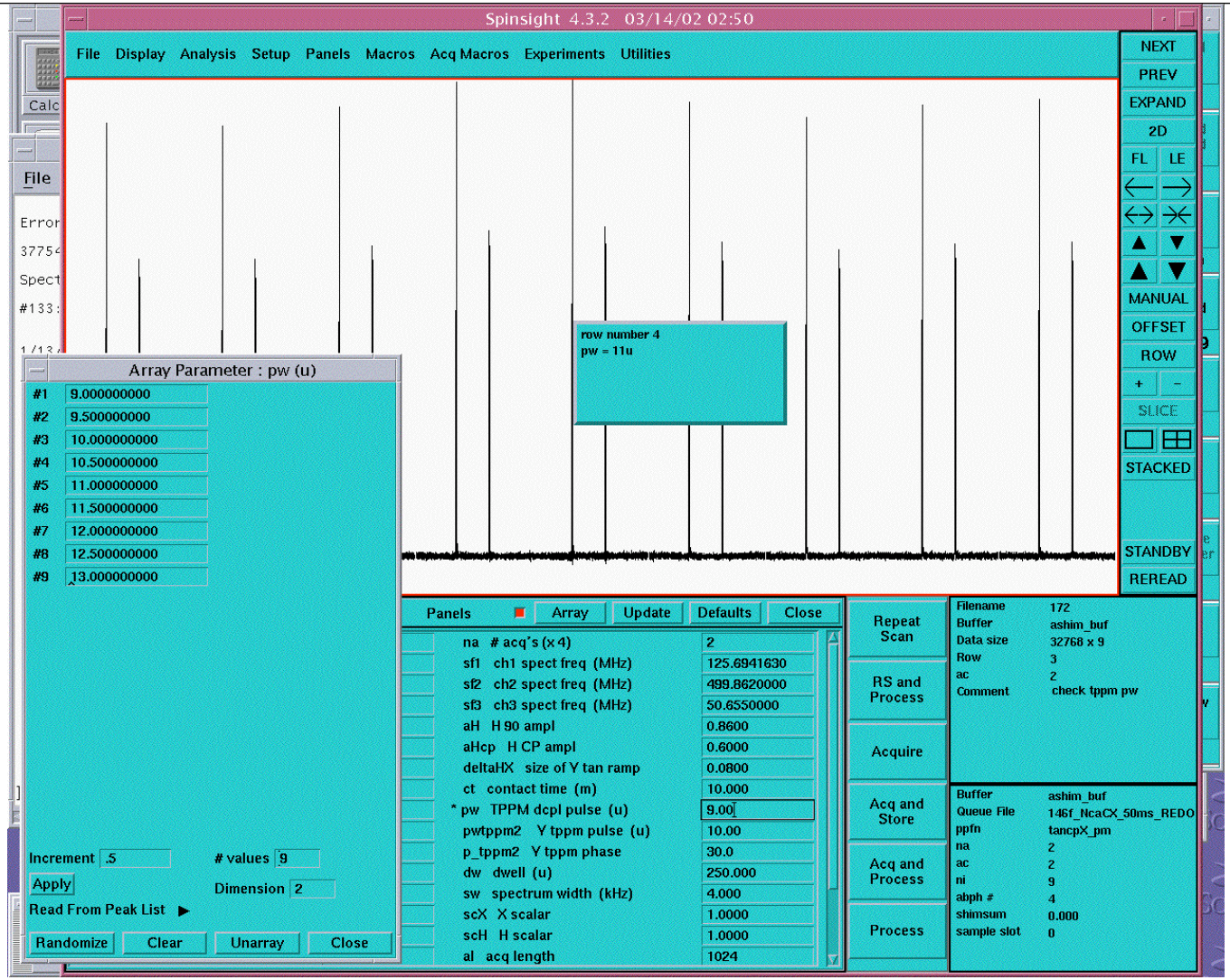
Notice now that when acquiring for 200 ms, the decay in the last 100 ms is greater, but in the first 50 ms it has been reduced. This is more accurately assessed by looking at the frequency domain, where the foot is now only about 20 Hz wide (whereas it was 30-35 Hz before). We are approaching this probe's limit of shim quality (due to material susceptibility).



Now the final step in the shimming procedure is to “touchup” with a longer window of 100 ms.

If the “normal” or “large” ranges are used, the potential exists for the autoshim algorithm to chase the narrow component, and create a foot in the spectrum. We wish to avoid that. In most protein NMR, the acquisition times will not exceed 50 ms, so it is counterproductive to emphasize the very narrow component of the adamantane signal. Here is the result.

(Notice that decoupling amplitudes of ~40 kHz are more than sufficient.)



Even with a very easy-to-decouple sample like adamantane, near the final stages of the shimming procedure it is useful to optimize the TPPM settings. We'll return to this issue in more detail later, but for the moment recognize that the peak height has a strong dependence on the TPPM pulse width with fixed angle (**p2**) and decoupling amplitude (**aHdec**). A full 3D optimization might give further gains but is usually not necessary for adamantane.

Part Two:

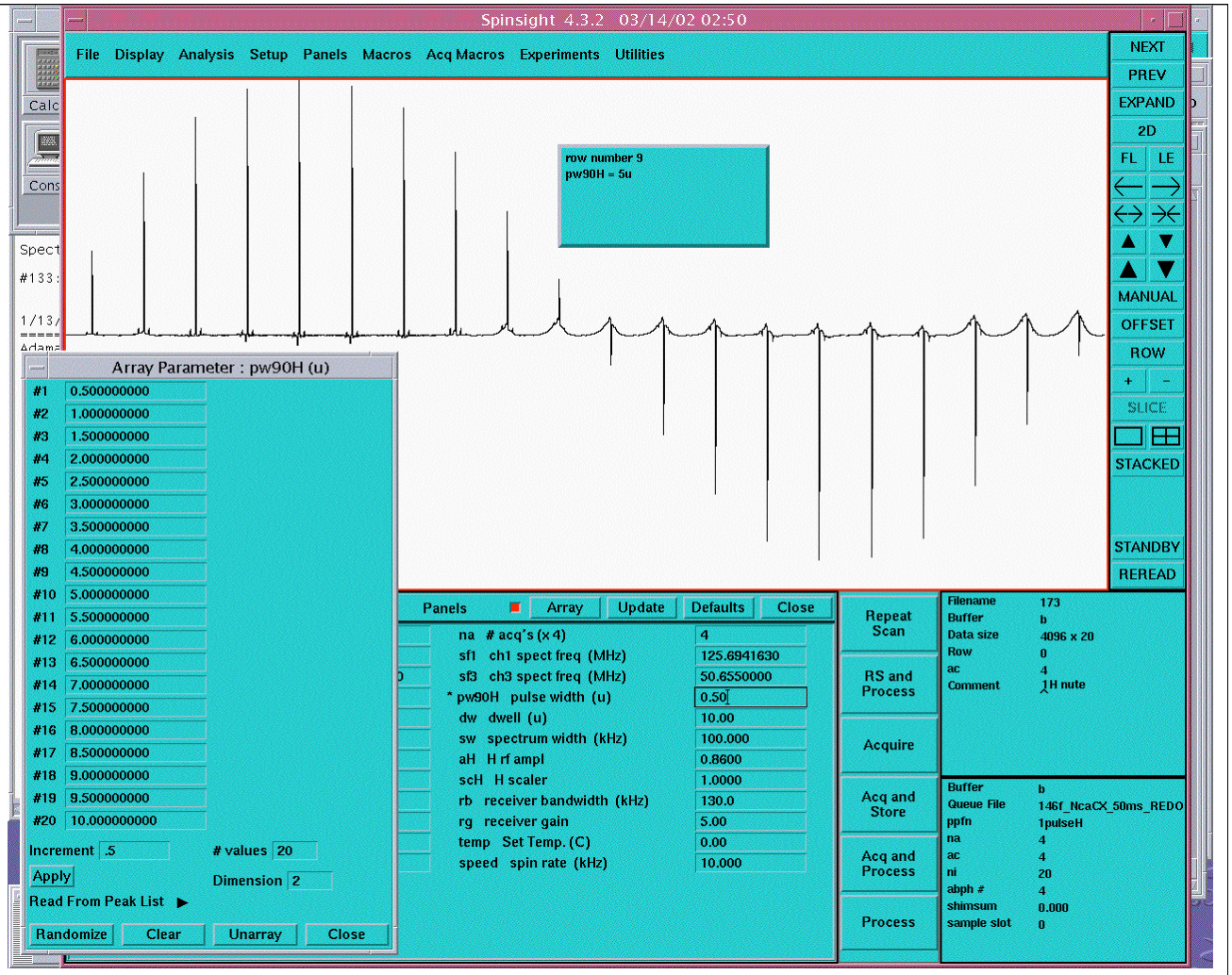
Basic ^{1}H and ^{13}C Calibrations

Sample:
80% Adamantane + 20% KBr
(physical mixture)

Anticipated Time Requirement
Complete setup: 20-30 minutes

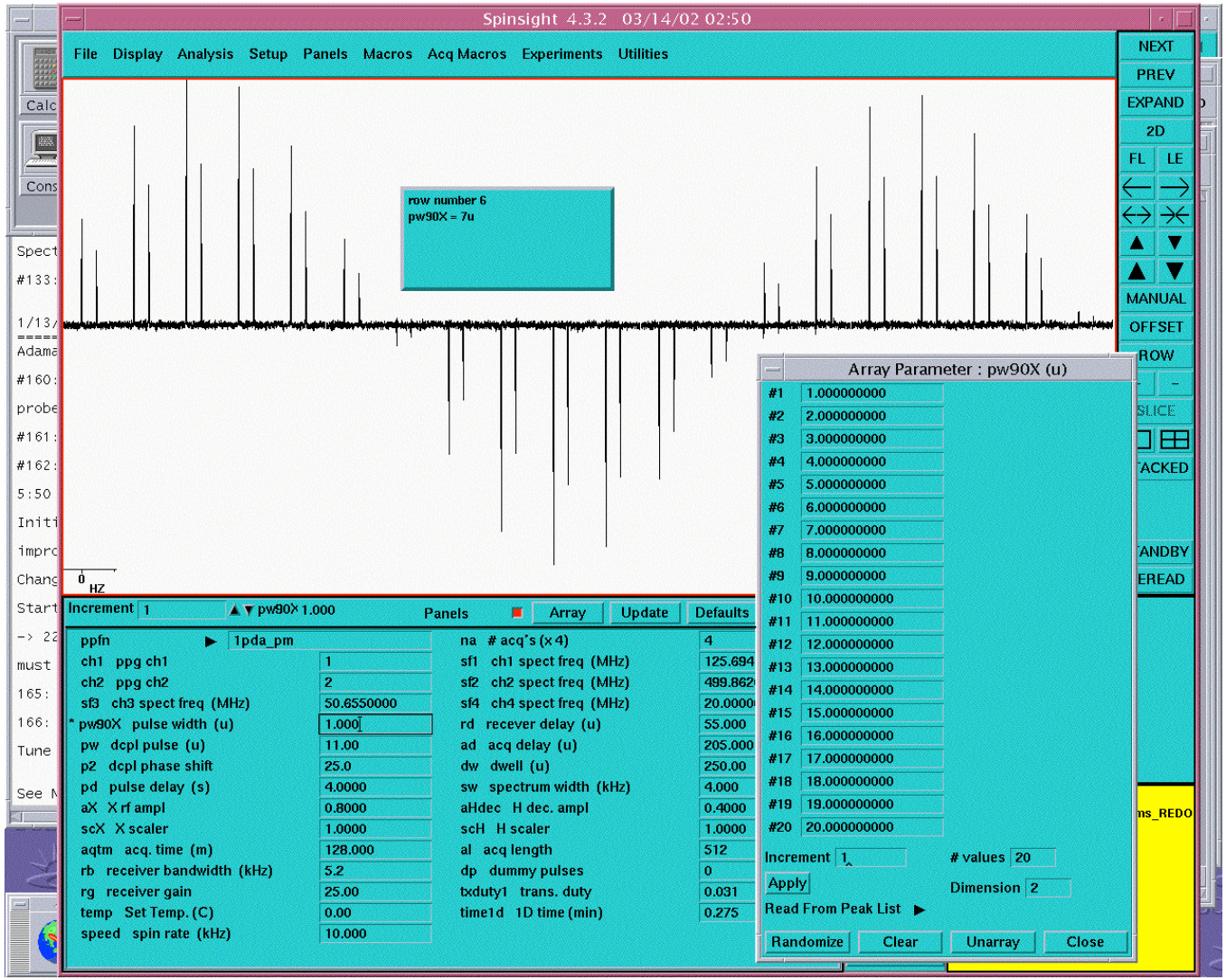
At this power level (aH = 0.86), the field is ~100 kHz (based on a π pulse slightly greater than 5 us). This implies a field at full power of ~100/0.86 = 116 kHz, which is slightly low but ok.

Notice that the probe has a significant 1H background signal from the polyimide materials (torlon spacers and drive tip).



Typically before shimming, you would already have a good idea of the pulse widths, CP and decoupling conditions. This next section assumes you have not yet calibrated these parameters. In practice, this should be done before shimming. The first and most important test to conduct is a rough measurement of the ^{1}H field strength by a short pulse nutation as illustrated above. For a 3.2 mm probe @ 500 MHz, the field at full power should be ~125 kHz or less. If the field is much higher than this, add attenuation at the transmitter output, to avoid probe damage.

Note that the B_1 homogeneity of the probe can also be estimated (as well as the quality of centering the sample in the rotor) from the I450/I90 = ~85±5% (a smaller increment of pw90X would be required to determine this more precisely.)

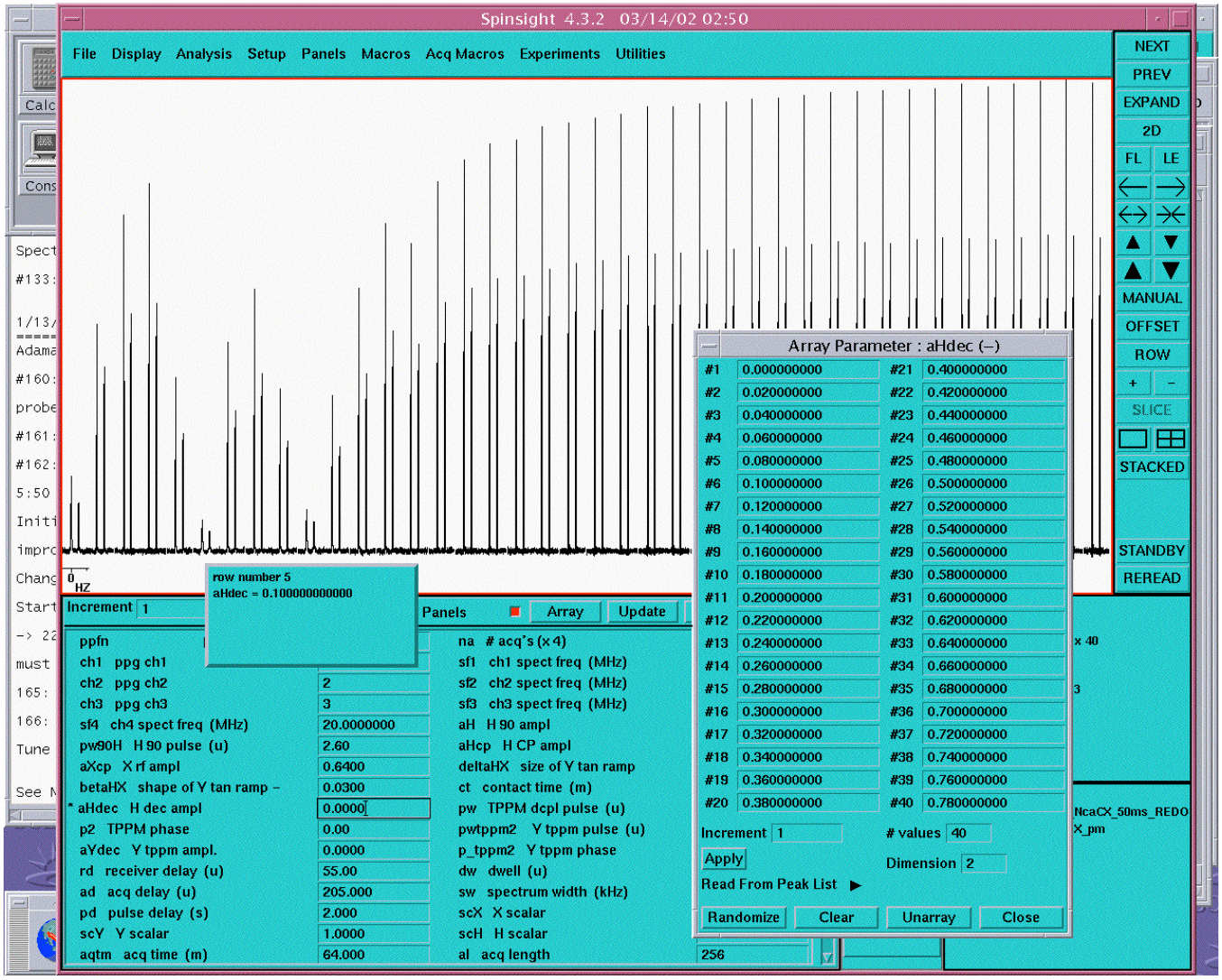


Likewise the ^{13}C pulse width should be tested next. With a 3.2 mm probe @ 500 MHz ^{1}H frequency, the peak ^{13}C field should be about 100 kHz. At aX=0.8, the π pulse is 7 us, corresponding to a field of ~71.4 kHz / 0.8 = ~90 kHz at full power. This is ok.

n=1 @ 0.10

n=2 @ ~0.18

The field is not perfectly linear in aHdec because of the ^{1}H tube amplifier response function.

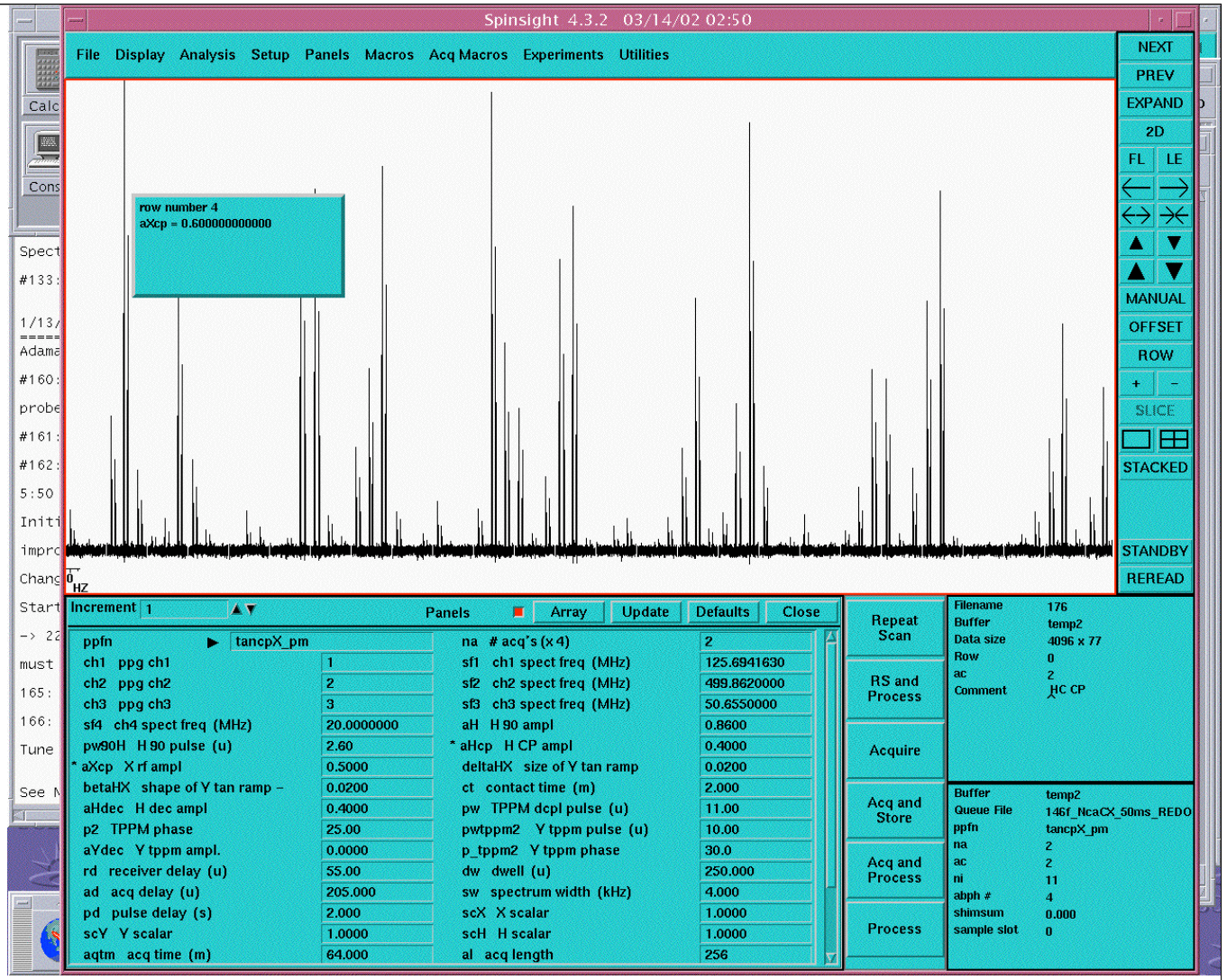


Rotary resonance recoupling (R3) conditions on the ^{1}H channel are easy to measure with adamantane and useful for more sophisticated setups later. Here the aHdec value is arrayed in a CP experiment; the peak intensity of the ^{13}C signal dips when R3 conditions are encountered.

At each aHcp level, there are two aXcp match conditions (n=±1). Because the conditions are rather narrow in adamantane, we use a small ramp (deltaHX = 0.02) on the ^{13}C channel.

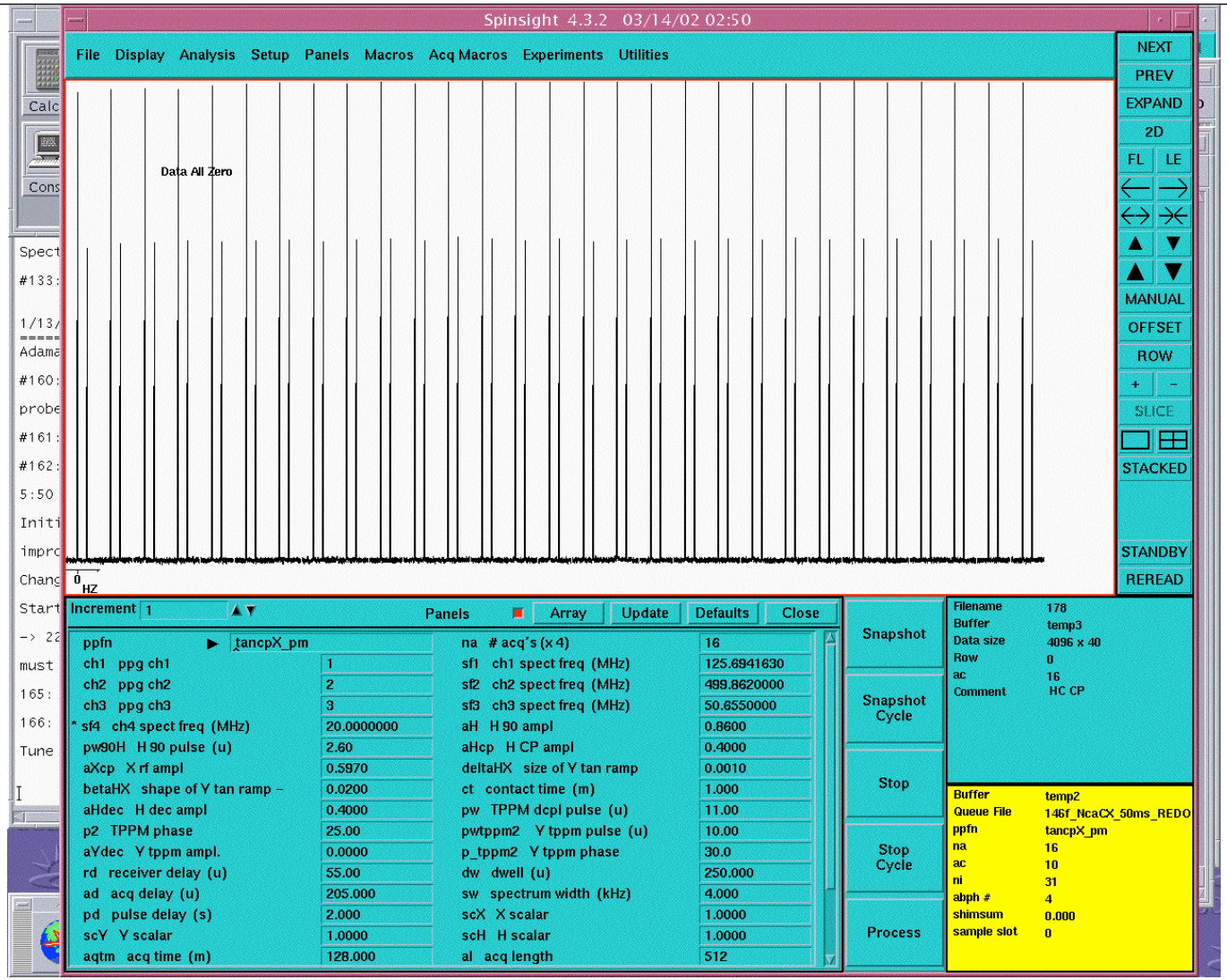
In this case the relatively low amplitude of aHcp = 0.40, aXcp = 0.60 (note: Spinsight does not display the arrayed parameter in the cursor box when it is at its initial value).

Notice that this corresponds to ~45 kHz for ^{1}H and ~55 kHz for ^{13}C, based on earlier calibrations. Large, half-integer multiples of the spin rate usually give the best H-C and H-N CP conditions.

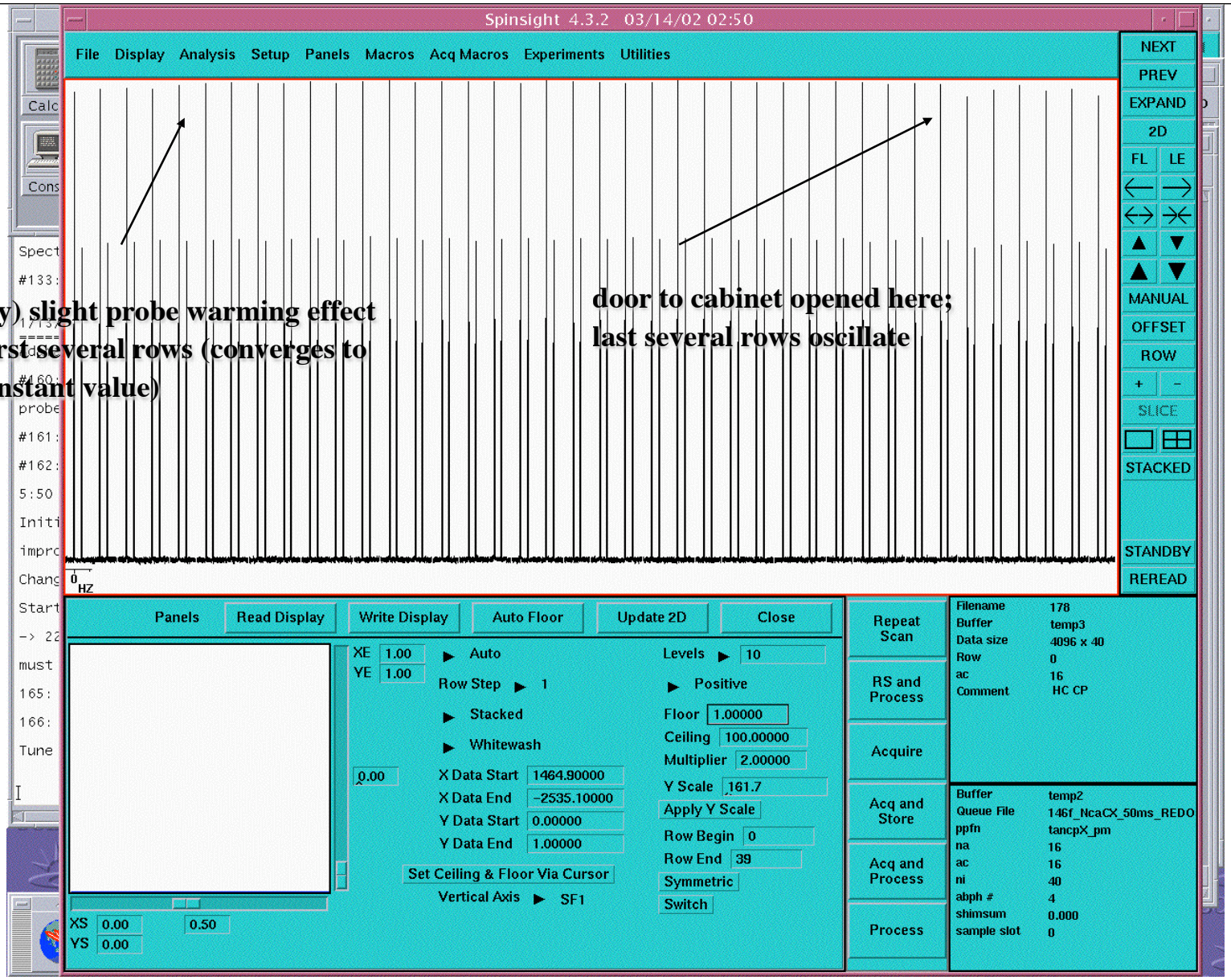


Now we search out CP conditions where the ^{1}H field is ~50 to 75 kHz, and the ^{13}C field is matched to an n=1 sideband condition. This is achieved by arraying the value aHcp in the third dimension from 0.4 to 0.7 in steps of 0.05, and aXcp in the second dimension from 0.5 to 0.75 in steps of 0.025. This coupled dependence is used to find the best overall CP condition (which avoids R3 conditions on both channels and minimizes T1rho relaxation).

This is a good experiment to run over a coffee or lunch break, to test if your instrument is ready for more challenging work.



After fine-tuning the aXcp value (not shown), we set the deltaHX to 0.001 (effectively a constant amplitude CP) to test the adamantane CP stability for ~30 minutes (1 spectrum per 30 seconds). The stability here is quite good, with fluctuations of only ~2% (maximum excursion). This is due to excellent room temperature stability in Noyes 55, and the fact that the amplifiers are thermopadded and the cabinets closed. (Don't believe this? Look at the next page.)



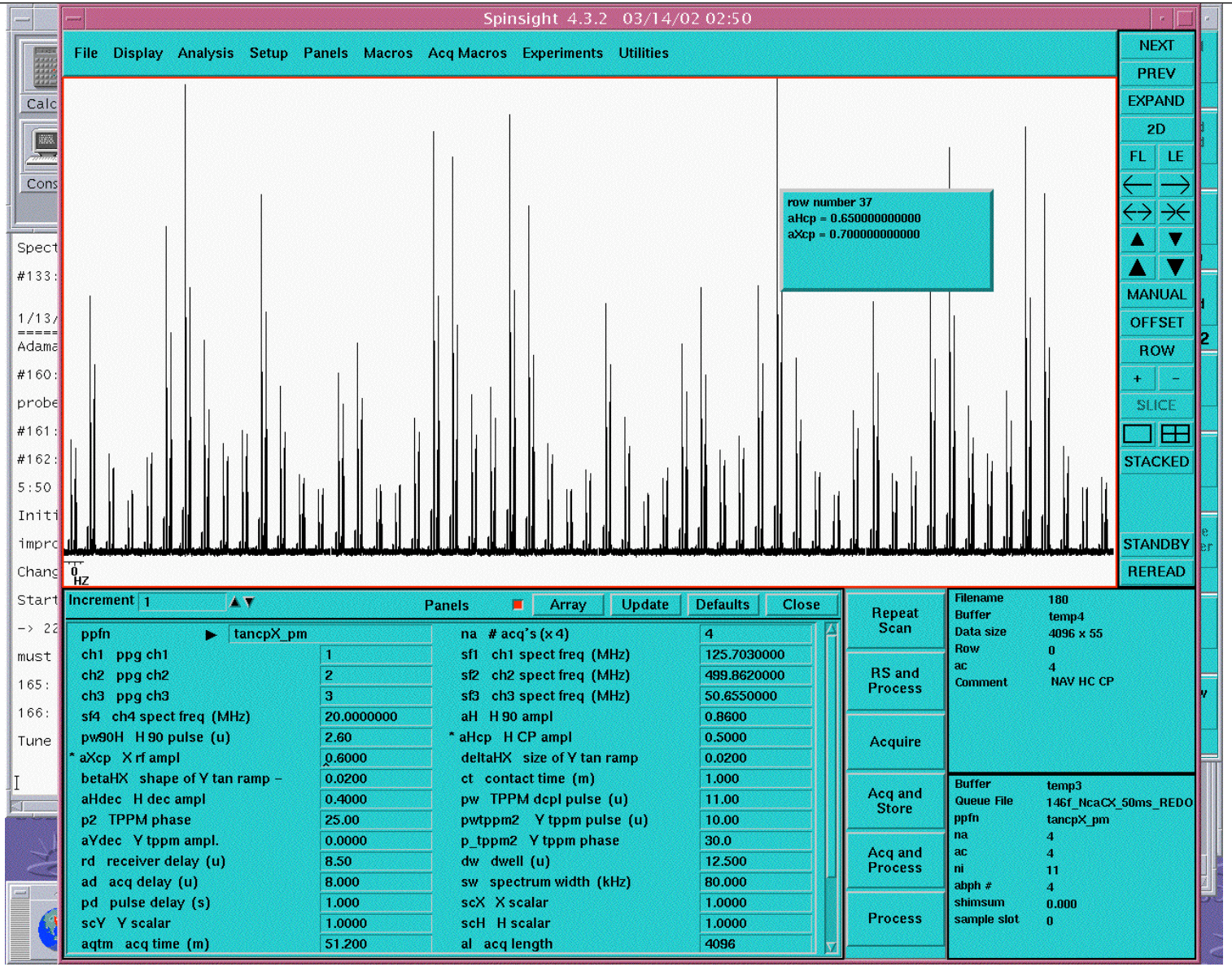
The door to the cabinet containing the 13C amplifier was opened while row 33 was being acquired. Rows 34 through 40 show much greater fluctuations (not just a shift in amplitude and

Part Three:
R3 and DCP Conditions

Sample:
U-^{13}C,^{15}N-N-acetyl-valine (NAV)
(21% in natural abundance)

Anticipated Time Requirement
Complete setup: 1-2 hours
+ overnight optimization
Refresher: 1-2 hours
(to find known CP conditions again)

Notice that there are a two conditions that are close to the same quality; with this probe (a bullet-proof, fixed frequency HCN balun probe), we select the higher power level. If the probe is suspect, use lower power.

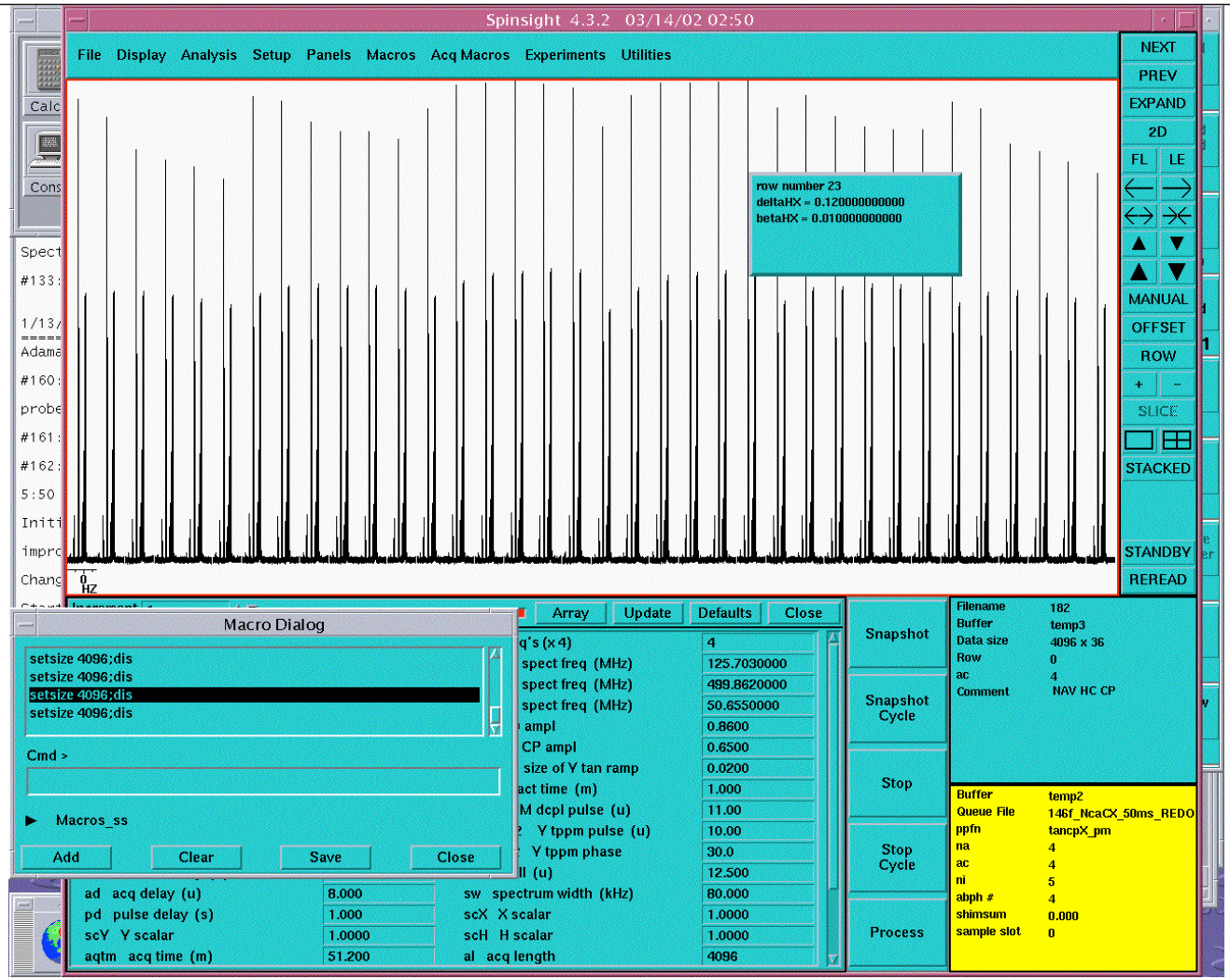


First check the ^{1}H-^{13}C CP condition with a 2D array of aHcp (from ~0.5 to 0.7) and aXcp (from 0.6 to 0.85). (We use a default spin rate of 88.8 ppm for ^{13}C, e.g., 11.111 kHz on the 500 MHz instrument.) For this "first pass" experiment, set deltaHX to 0.02 and ct to 1 ms.

We fine-tuned aXcp (not shown) to a precision of 0.005 before running this array.

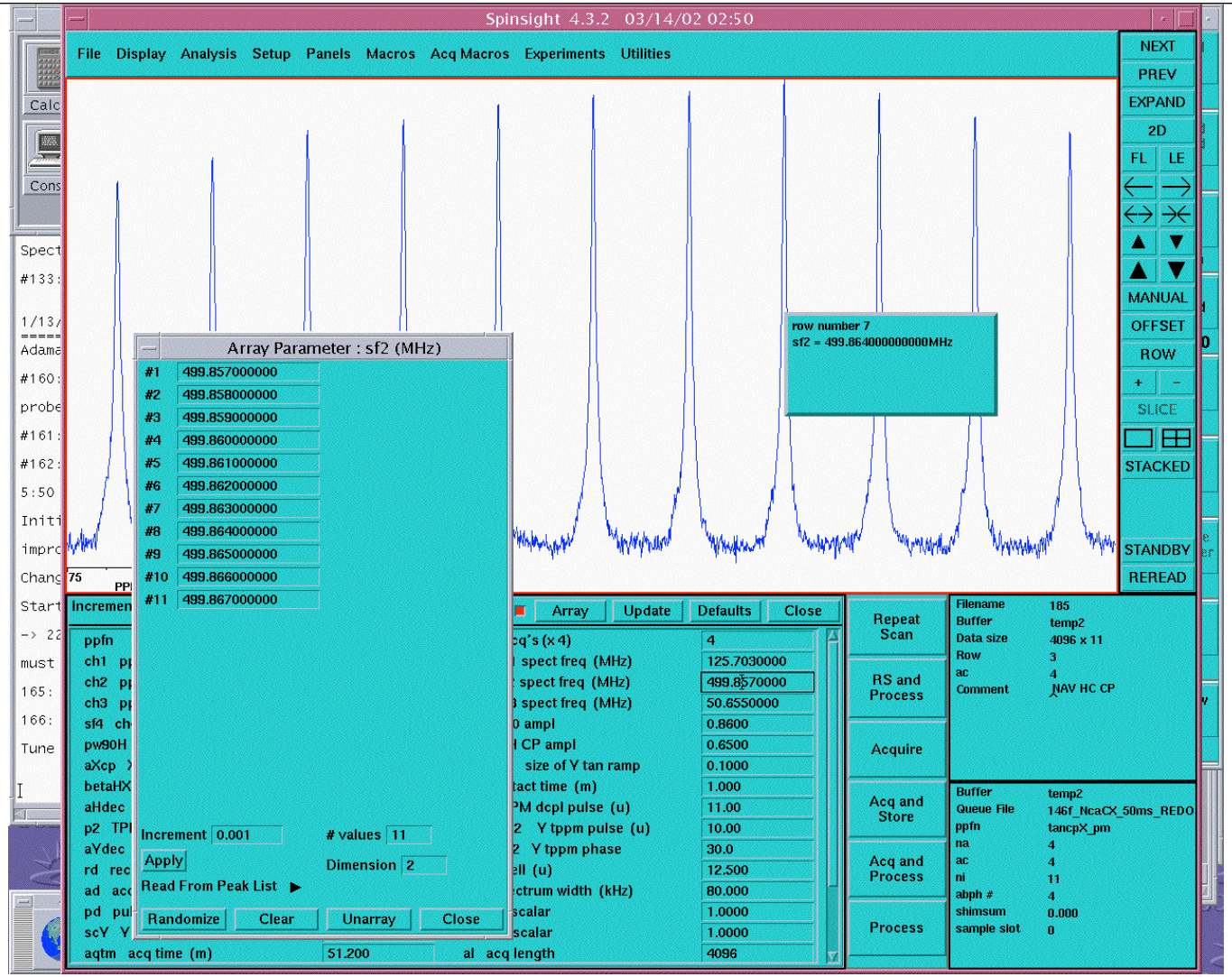
In the array pattern, first with betaHX at -0.05, deltaHX is varied from 0.02 to 0.12, then betaHX changes, etc.

The best values are found with relatively small beta.



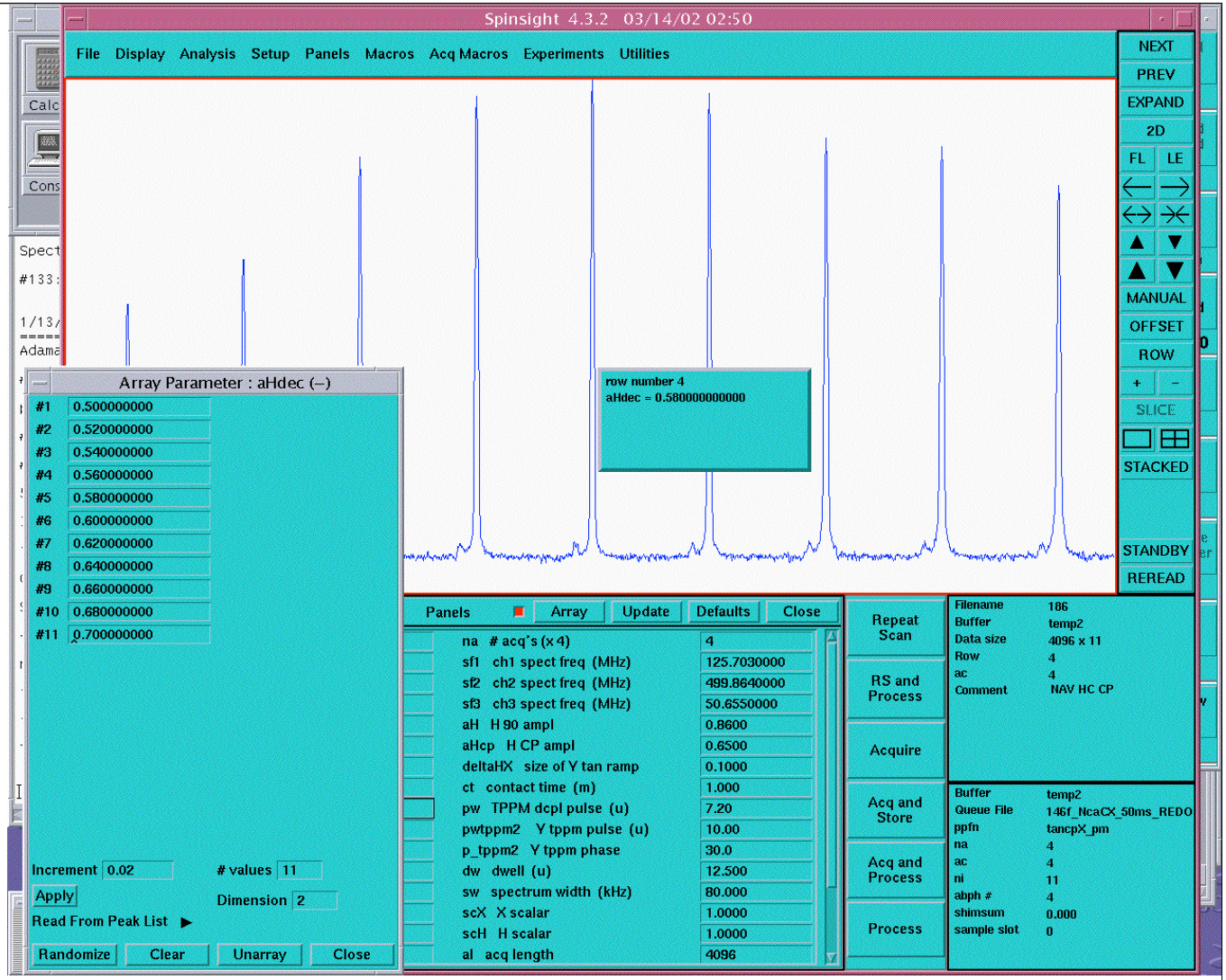
Next optimize the tangent ramp parameters deltaHX (0.02-0.12) and betaHX (-0.05 to 0.05), in 2D array. (A negative betaHX value corresponds to a ramp down in amplitude.) Here we see that a pattern emerges that for large (positive or negative) beta values, a small delta is favored, but for smaller beta's, larger delta's are favored, and the overall intensity is much improved. Furthermore, the result is stable (i.e., it does not vary dramatically with small changes), and we appear to have bracketed the optimal region, so it is not necessary to repeat the array with different values. We will, however, fine-tune beta a bit (to 0.012).

The effects are more dramatic as you go higher in B0 field, and/or to a methylene group. However there are still significant differences observed.

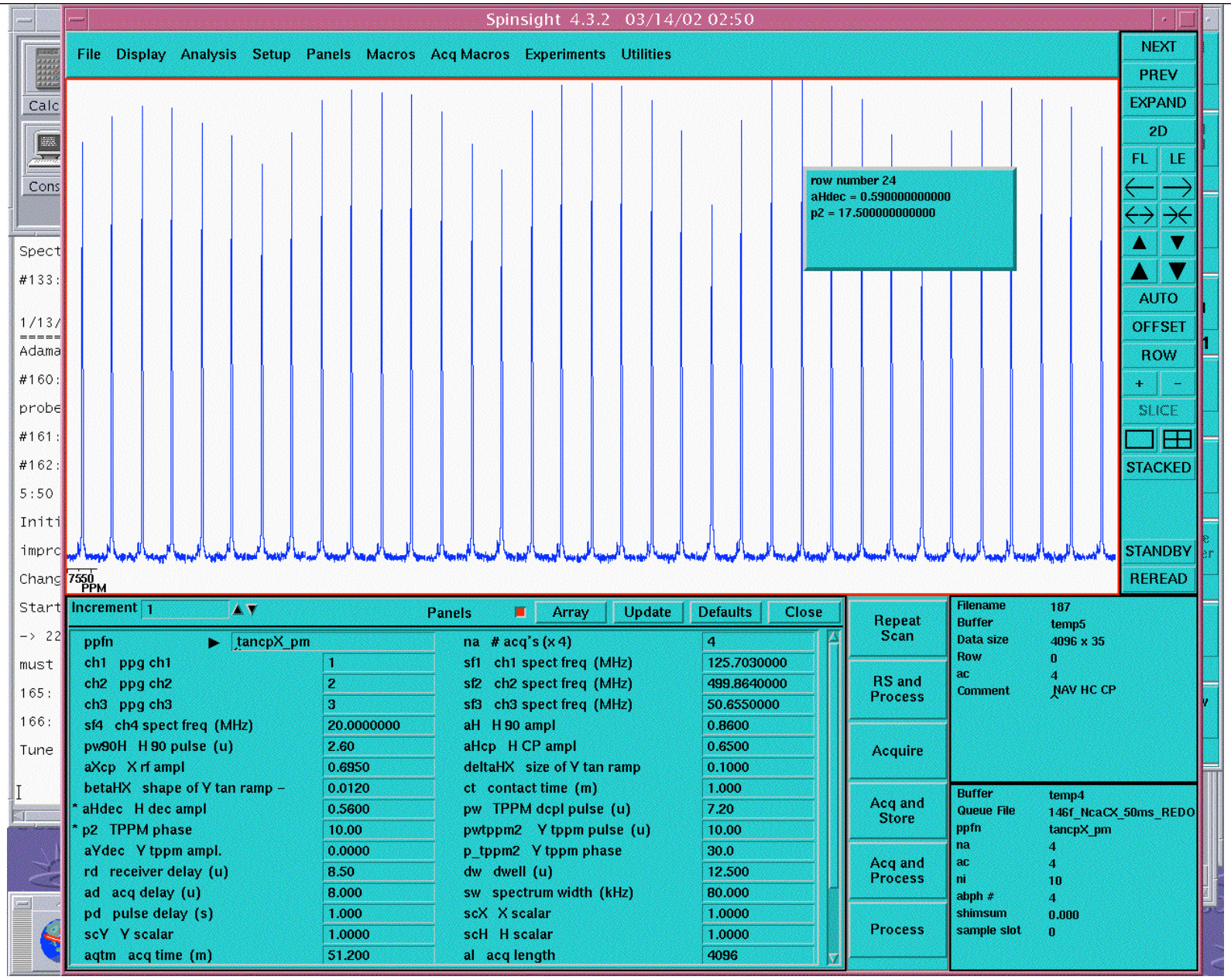


With a good ^{13}C signal, now is a good time to check the 1H decoupling. Previously we were using rather wimpy fields, which is why the methine CA and CB signals are so broad. First, we want to array the sf2 carrier frequency with a (relatively weak, ~50 kHz) CW field. Focusing on the region between ~45 and 75 ppm, we see the CA methine signal, and make a stacked plot. The trend is clear. If the trend is not obvious, decrease the aHdec value and try again.

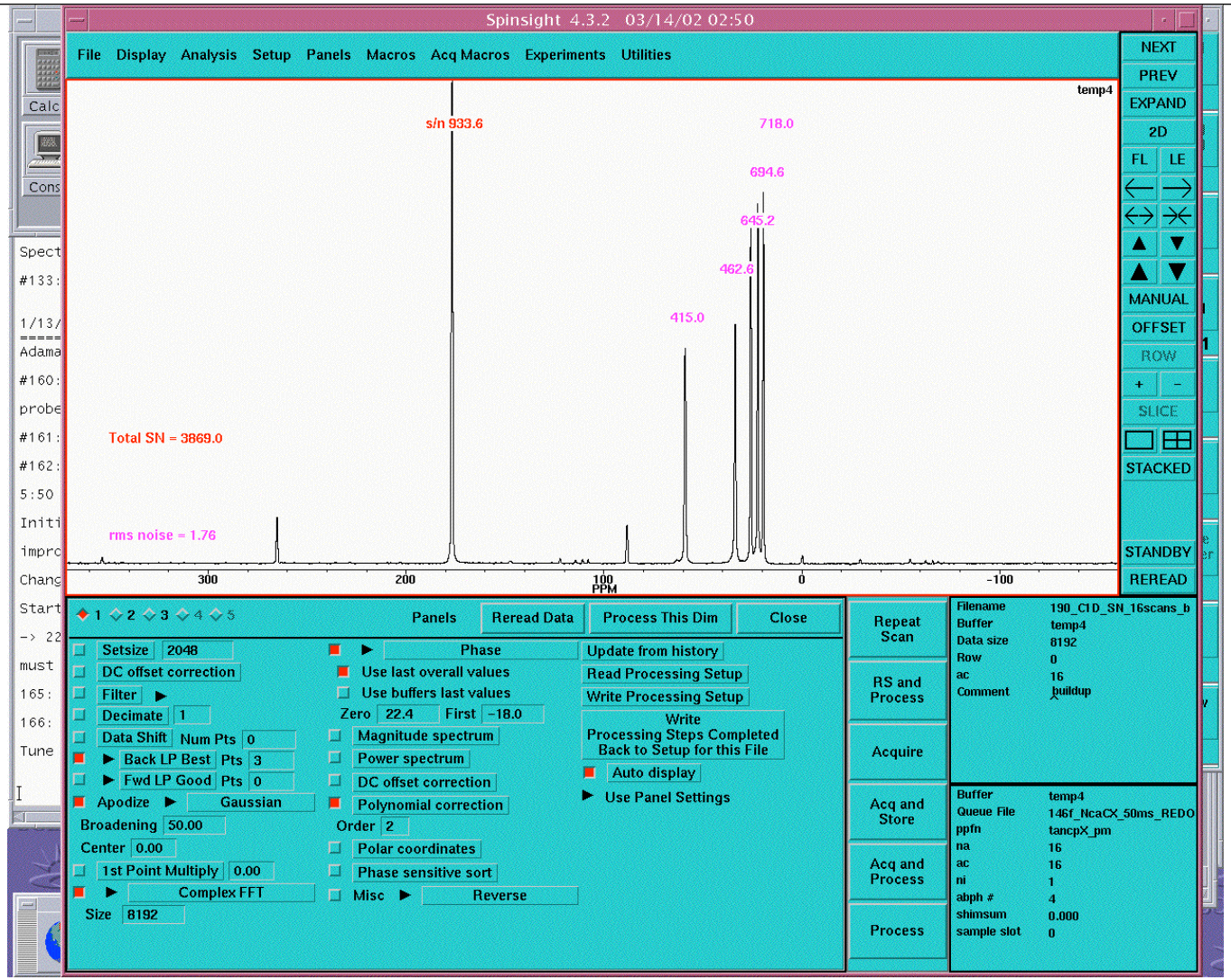
Again this result is not at all ambiguous, the way we like it.



Now set the TPPM condition. One could do a "blind search", but why waste the time? You know the pulse widths now, so take advantage of it. A good amplitude for TPPM in protein samples at 500 MHz is ~70 kHz, or a ~7.2 us switching time (close to a π pulse), with a 15 degree phase angle. We optimize aHdec with these assumptions for the TPPM parameters, and then come back to fine-tune the aHdec and p2 parameters in the next experiment.

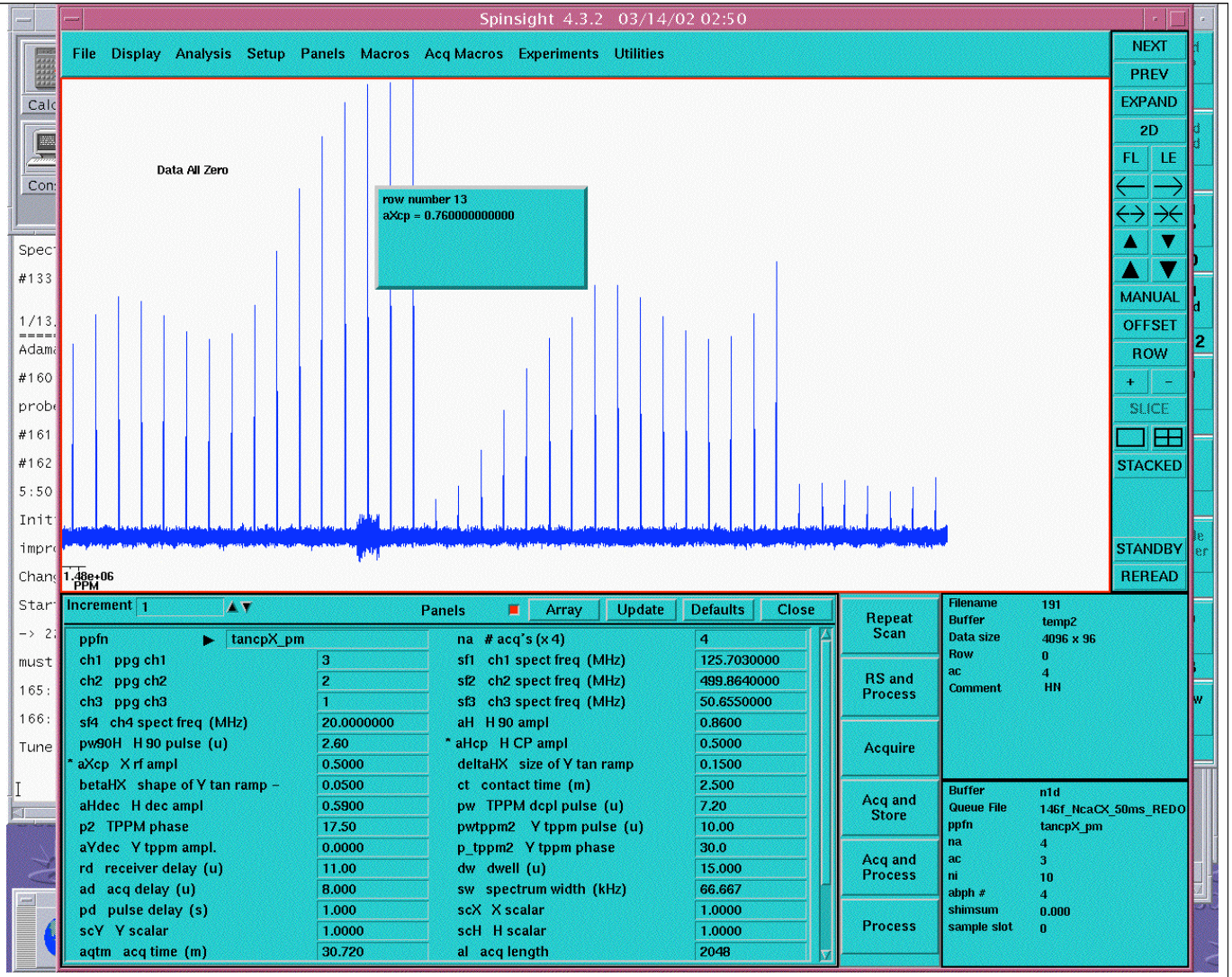


A 2D array of aHdec (±0.02 from previous) and p2 (from 10 to 25 degrees) gives some additional improvement.



We acquire a 16 scan reference spectrum, with a 3 second pulse delay (more than 5 times T_1) and 50 ms acquisition. Processing it with 50 Hz GB yields our standard group sensitivity standard. A Spinsight macro is used to add up the signal to noise ratios for all peaks. This number does not strongly depend upon decoupling conditions, but if CP is improperly set, or there is a hardware problem, the value will deviate significantly from the standard condition. Usually this is repeated 5 times and an average S/N reported. Check your value against the known value for this probe configuration. Does it agree within error?

The increase in noise level seen at aXcp=0.76 is very likely due to a corona discharge initiated on the LC trap circuit and/or the series capacitor which has the largest voltage across it at the ^{15}N frequency of 50 MHz. Although this pulse does not remain on during acquisition (when the arcing occurred), it likely arose from the ionized air around the capacitor. Bottom line: turn down the ^{15}N channel power, since the arc occurred soon after we started turning the ^{15}N power up.

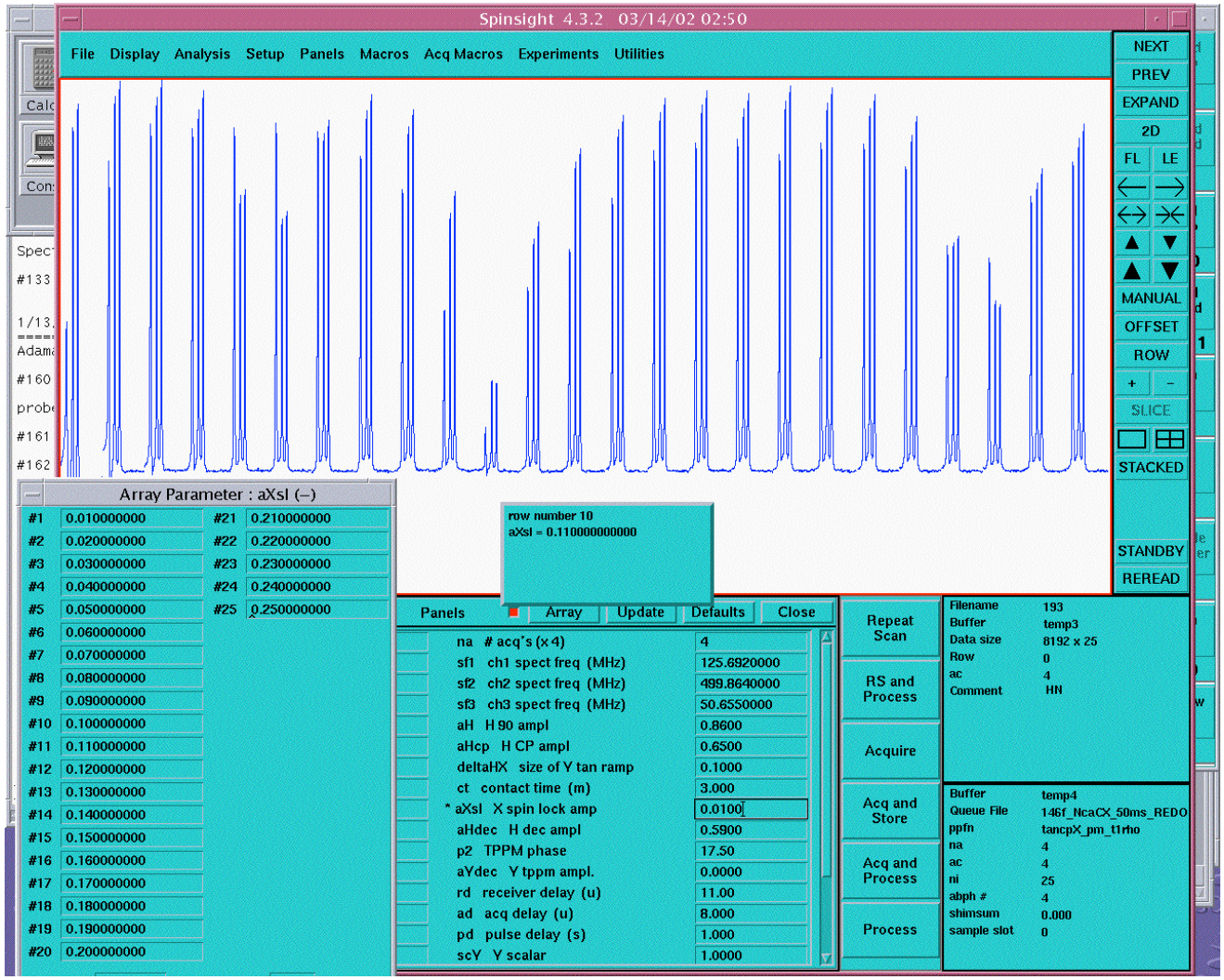


Optimizing HN CP by the same protocol... we'll skip most details other than to note that the array above proves the probe is not entirely bullet-proof, and that at higher aHcp values (>0.5), the aXcp (where X is now CHN3, or ^{15}N) cannot reach the n=1 match condition. So we set aXcp to its highest "comfortable" value (0.15) and searched for the best aHcp, which turned out to be

n=1 condition is at aXsl = 0.11
n=2 @ 0.23

These values will guide our selection of DCP conditions later.

Generally we want to avoid the R3 conditions, since the effects of CSA and dipolar recoupling cause rapid loss of signal along the spin lock axis.



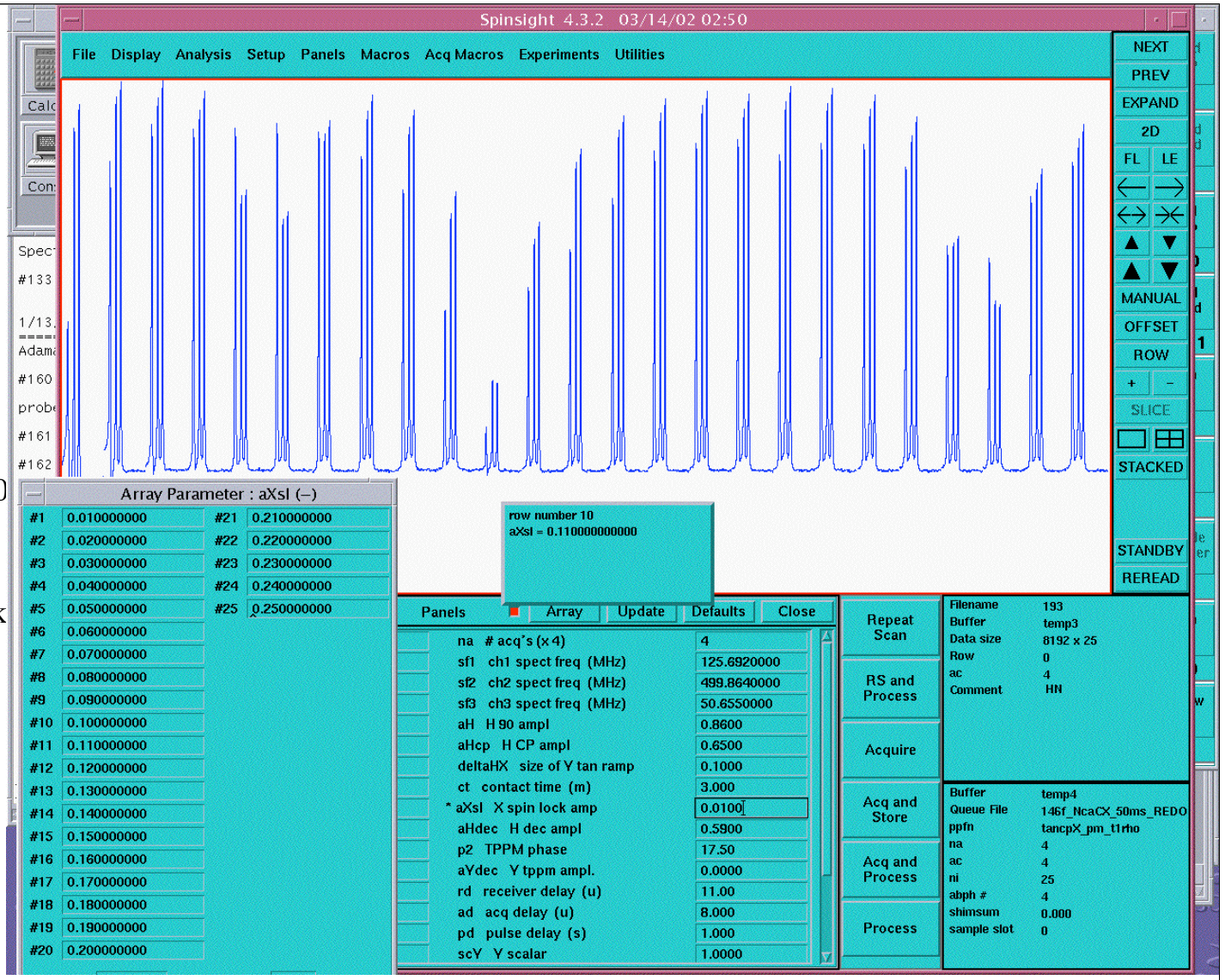
Next we measure the R3 conditions for ^{13}C and ^{15}N. For ^{13}C, the cleanest result comes from setting the carrier in the methyl region (with ppfn = tancpX_pm_t1rho), and arraying the parameter aXsl, a spin lock after the CP period, with a contact time of 1 ms.

n=1 condition is at 0.22
n=2 @ 0.43 or so

Note that (not by coincidence) the ^{15}N field in kHz is approximately half of aX*100.

The ^{13}C field in kHz is approximately equal to aX*100

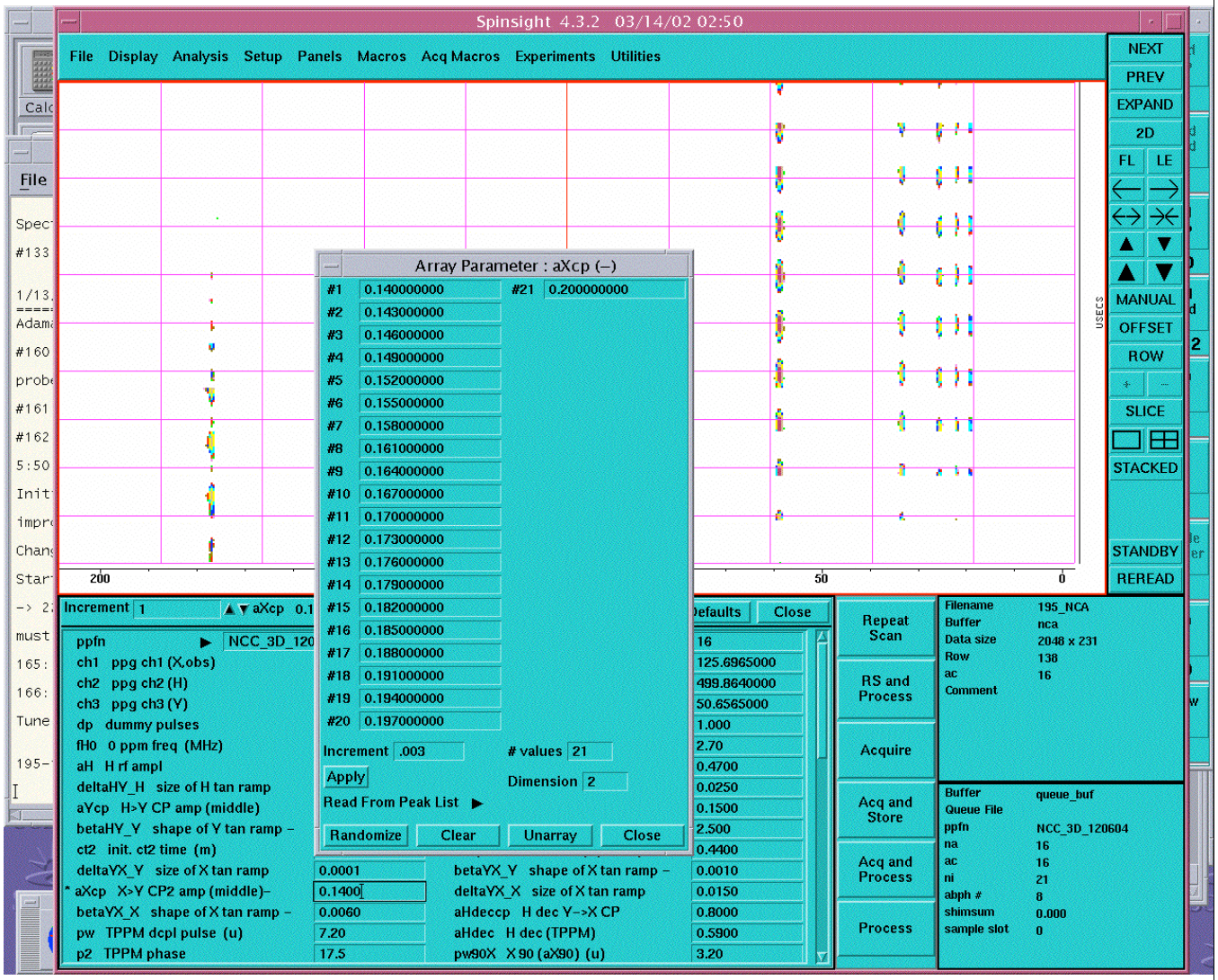
This makes subsequent calibrations a lot easier to think about.



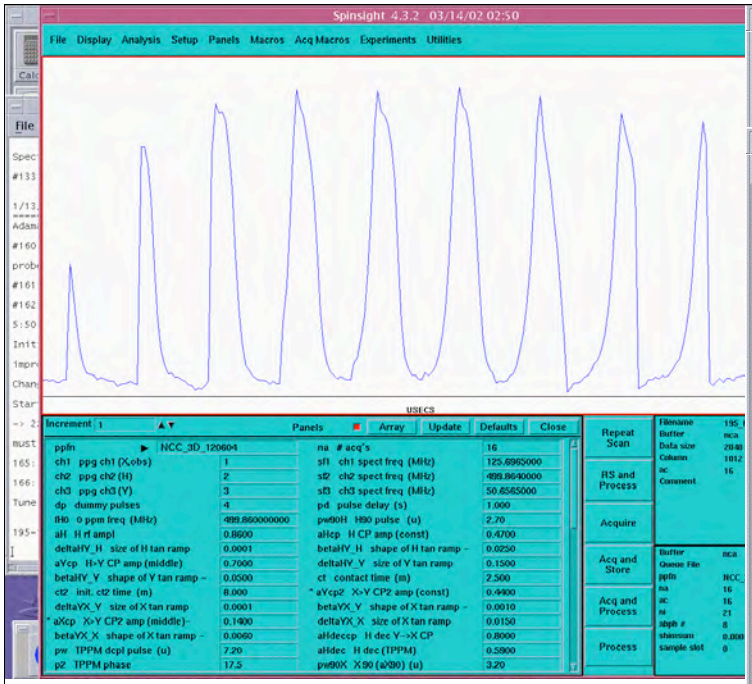
For ^{13}N, we use the one and only amide signal in NAV.

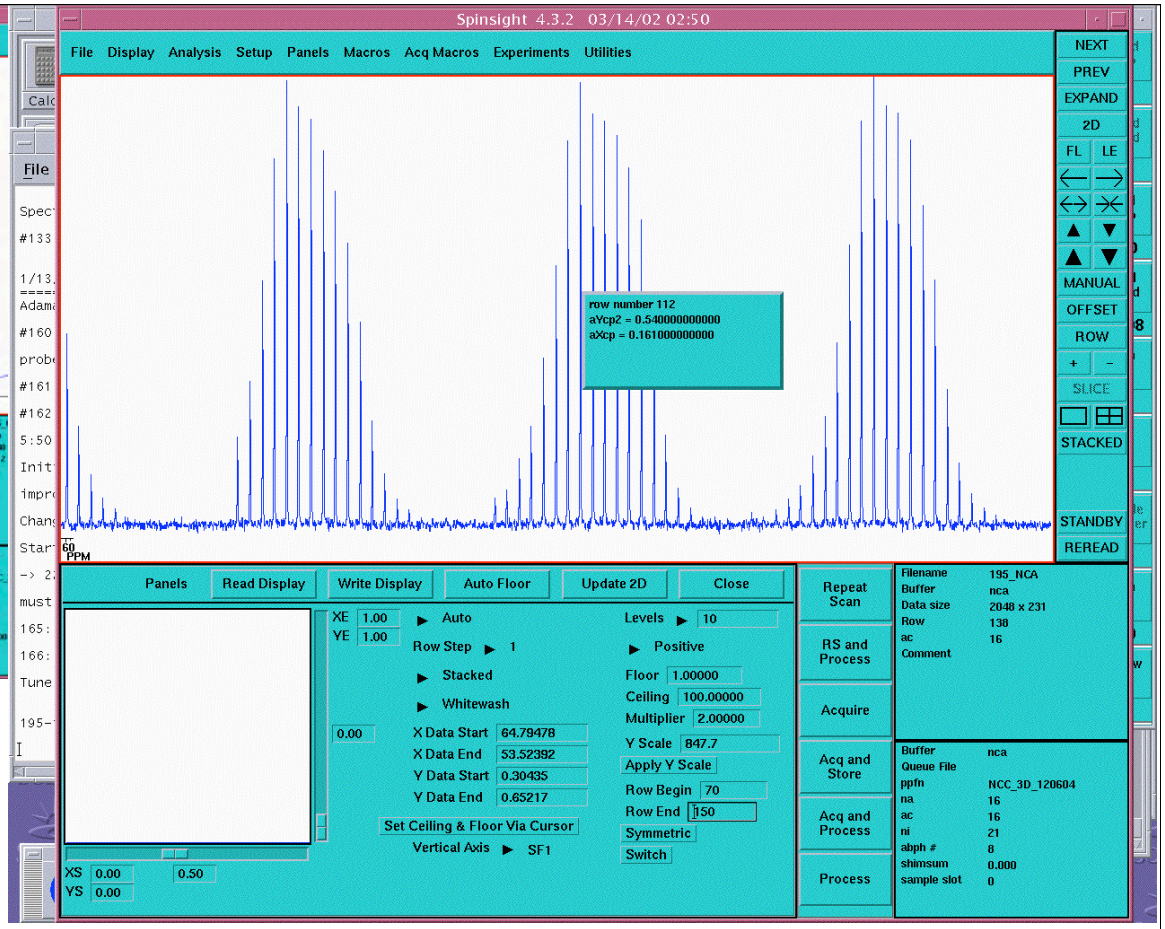
A clear pattern emerges from the data: the best condition is found where wN and wC are very close to half-integer multiples of the spin rate (wR).

From here we can fine-tune, but experience shows that at this spinning rate (11.1 kHz) on a 500 MHz instrument, there are no other generally applicable NCA conditions worth pursuing.



We search for the N-CA SPECIFIC CP condition (Baldus et al, Mol Phys 1998) by arraying aYcp2 over the range where wN = ~5/2 wR, and aXcp so that wC = ~3/2 wR.. This avoids R3 conditions while minimizing the wC field, thereby maximizing the ratio wH/wC (see papers by Ishii, Bennett etc. ~1997-1998)

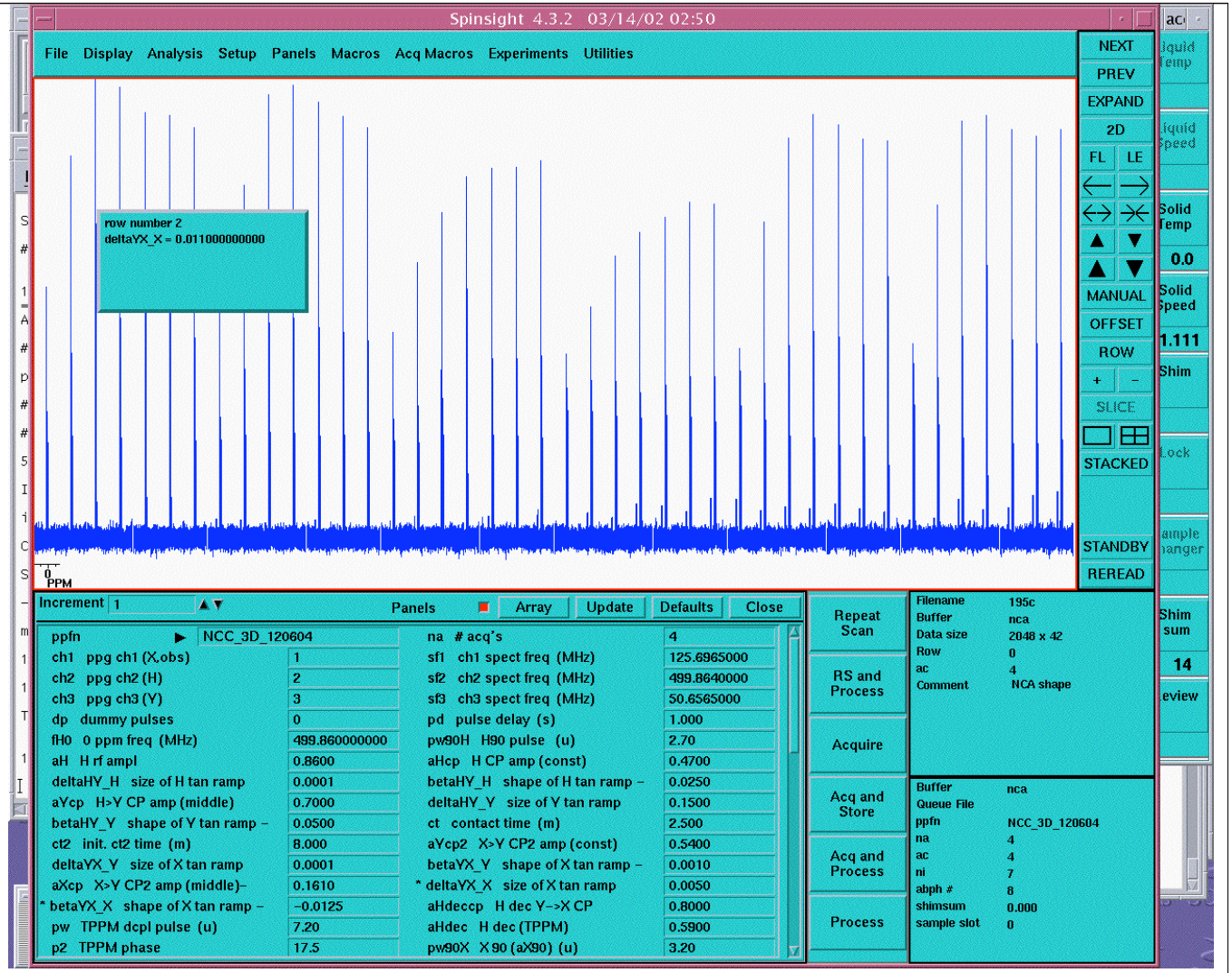




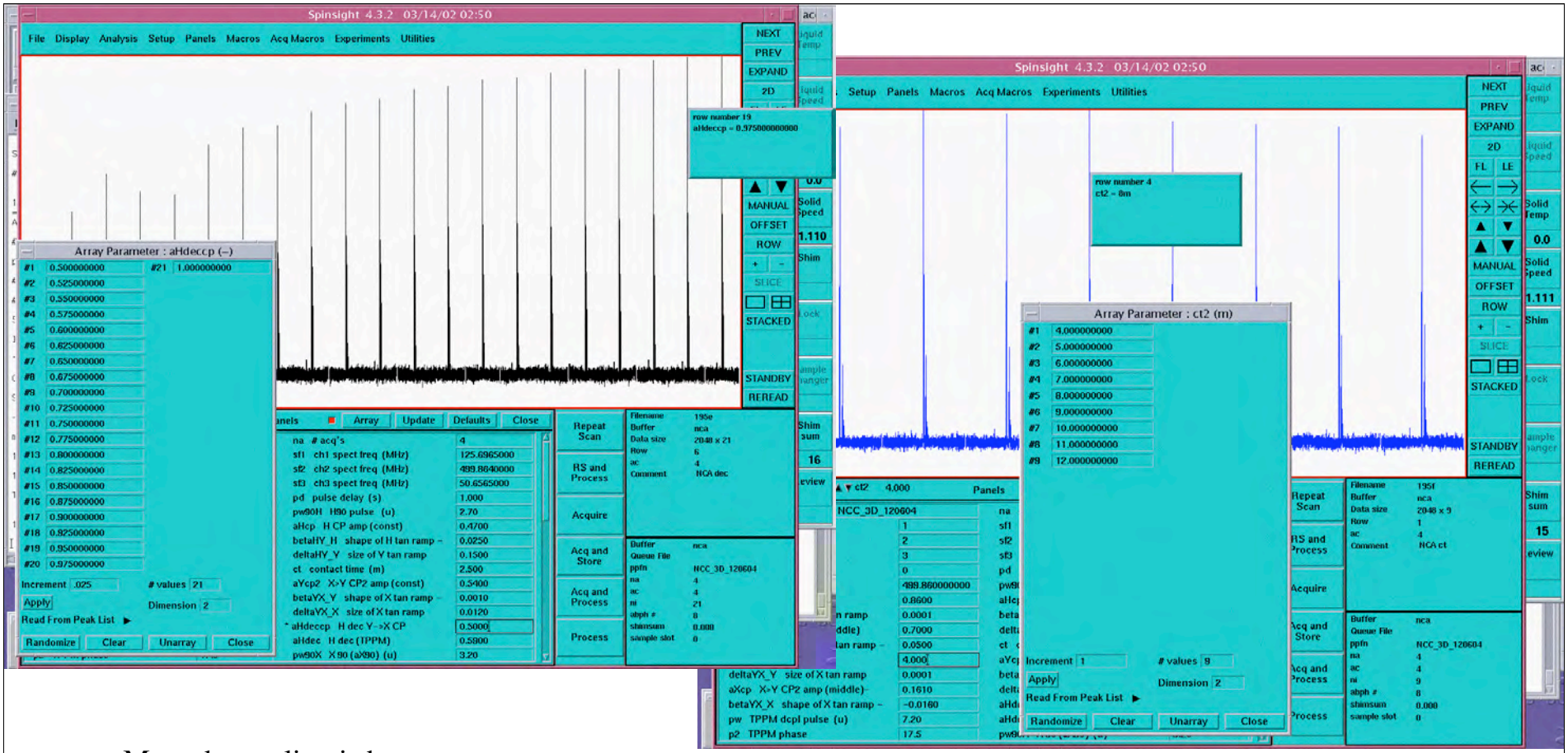
The peak CA signal is 316 in 8 scans (pd=1), with the reference signal (16 scans, pd=3) of 1460. So the efficiency is 316/1460*2 = 43%. (This is the lower bound, because the NCA spectrum has too short of a pd. We'll check more precisely later.)

Here we examine the CA resonance in more detail, as either a slice through the appropriate column (upper left), or as a stacked plot of the CA region. Both presentations show the pattern where the best overall condition is at ~aYcp2=0.54 (wN = 5/2 wR), and aXcp=0.16 (wN = 3/2 wR). We could proceed to examine other conditions—e.g., wN = 5/2 wR, wC= 7/2 wR—but conditions with higher wC fields will suffer from insufficient wH decoupling, and in most cases the probe will have difficulty achieving much higher wN fields. We fine tuned the exact value of aXcp (not shown) to be precisely 0.161.

The value of beta = -delta gives a tangent shape that is approaching linear (beta>>delta is perfectly linear, beta<<delta gives a tangent with most of its amplitude change occurring at the very edges of the shape).



We fine-tune the deltaYX_X and betaYX_X parameters (in a coupled 2D array) to optimize shape (upper left): it shows clearly that a negative beta value is preferred (ramp down on wC, to minimize loss due to insufficient decoupling). Then we individually test aHdeccp and ct. These are coupled somewhat, but generally their choice is limited by sample and probe characteristics (more is almost always better within the limits of probe performance for this experiment).



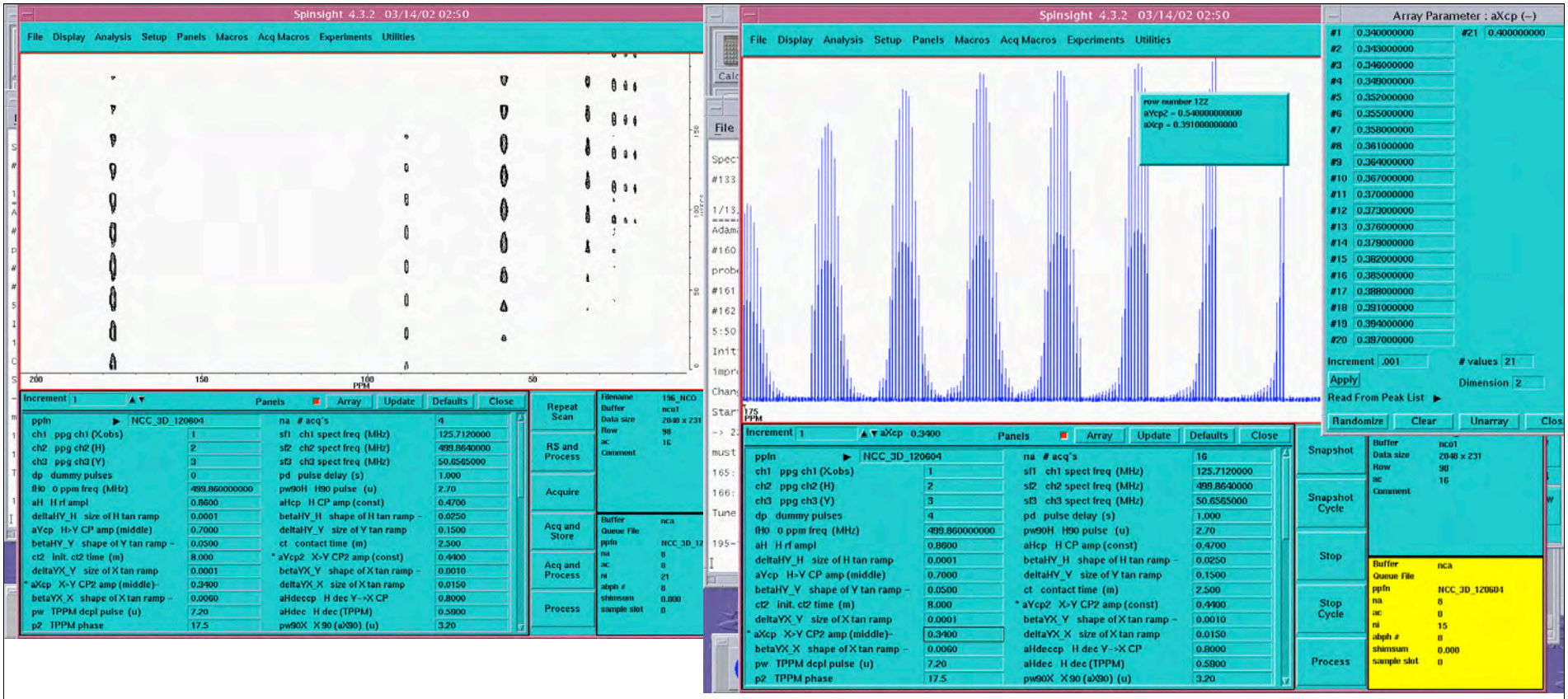
More decoupling is better...
until the probe burns up.
Then your experiment is done.
So we work up to full power,
but only after the transmitters
have been properly padded.

The buildup curve reaches its
maximum at ~8 ms; after that
point, polarization is spread to
the other aliphatic C signals or
lost to insufficient H decoupling

Here we show the arrays of aHdeccp and ct.

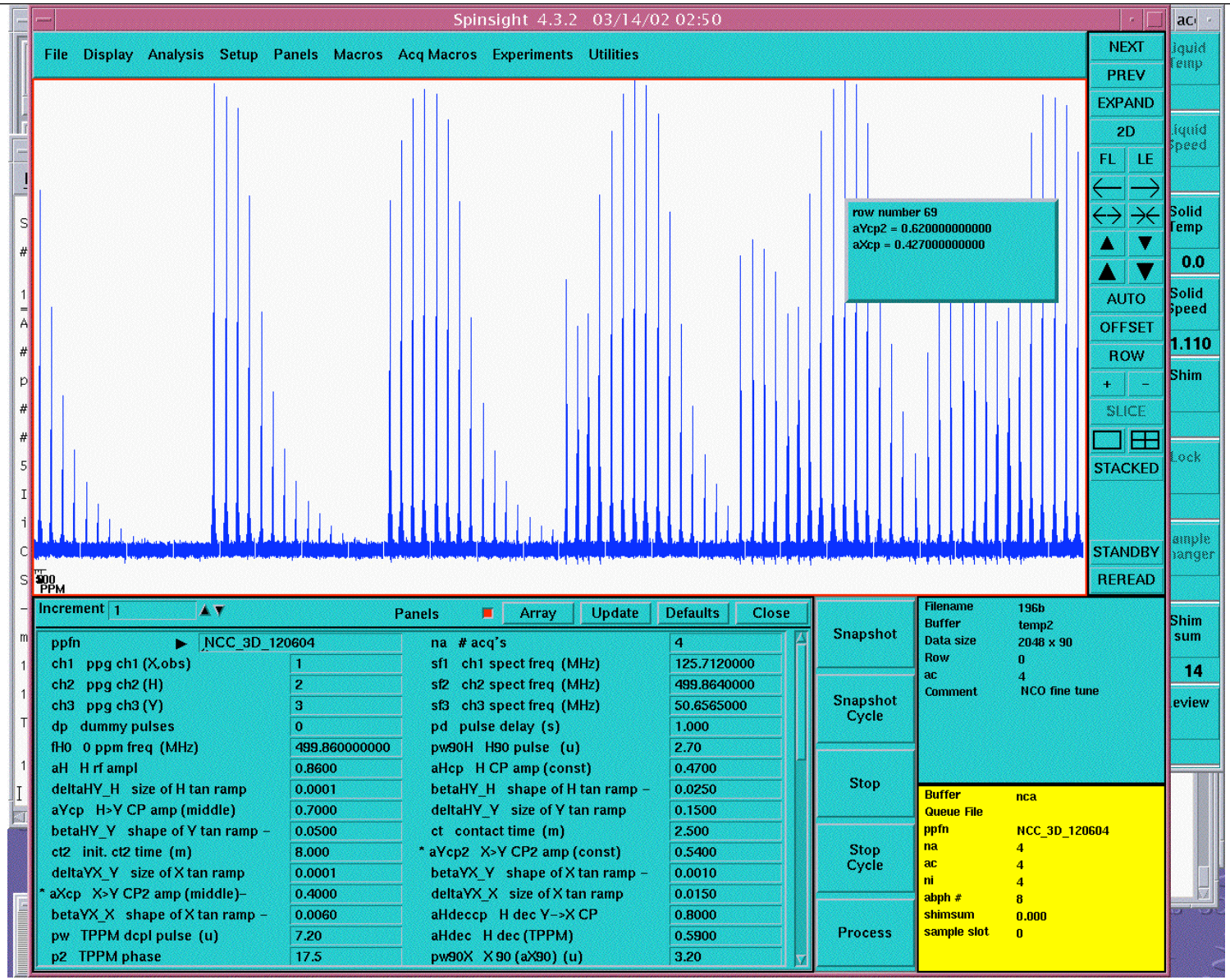
The best result gives an intensity of 891 in 16 scans (pd=3), or 891/1460 = **61%.**

This indicates that the HNCA condition is *very* well set (or the HC condition is badly set, but we know



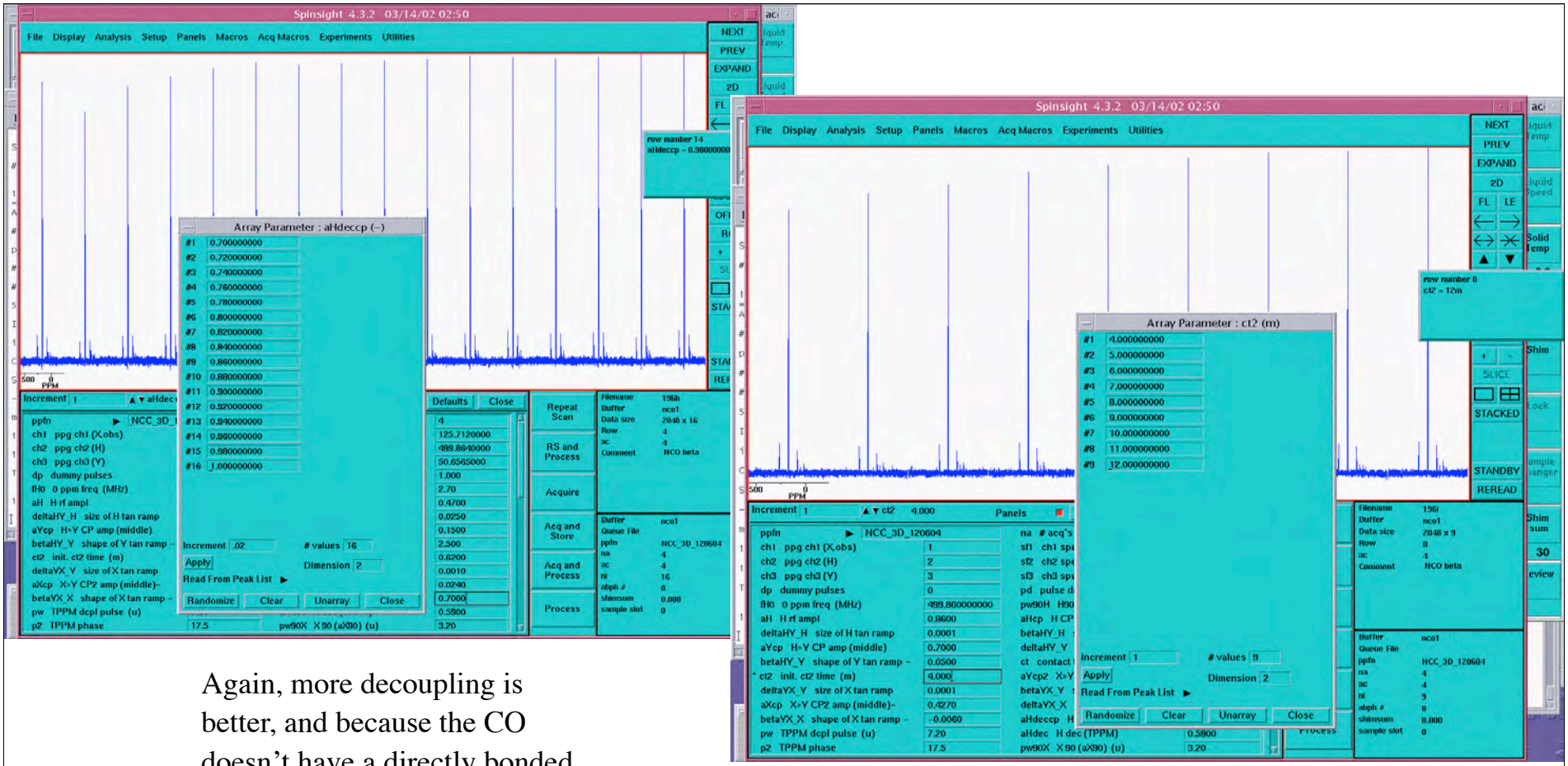
For NCO, we'll test two conditions. First: wN = ~5/2 wR, wC = ~7/2 wR. The higher wC field is better for CO (relative to CA) because there is no directly attached proton to the CO; thus H decoupling effects are less important (although not negligible).

Here we follow the same general strategy of arraying aYcp2 and aXcp around the appropriate values. On this first iteration, we did not go high enough in the aXcp value (note the asymmetry on the right side of the stacked plot). So we'll try again with slightly higher values.



Now we have a stable maximum as a function of aYcp2 and aXcp.

Note that this was optimized with aHdeccp=0.8, less than maximal.



Again, more decoupling is better, and because the CO doesn't have a directly bonded H, longer mixing times seem to work better. We stop at 12 ms

The resulting HNCO path signal has a peak height of 1714 and integral of 2.02.

The HCO reference has a peak height of 3289 and integral of 6.16.

So based on the peak height, we have 52% efficiency, or based on the integral we have 33%.

The discrepancy arises from NAV having two CO's, only one of which is directly bonded to a N.

So we should take the integrated value and multiply by 2, giving an overall HNCO efficiency of 66%.